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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLVIII *AUGUST 1959* NUMBER 8

Studies on the Adsorption of Odorous Materials I*

Surface Potential Changes of Solid and Liquid Adsorbing Surfaces

By J. O. KOPPLIN†, J. R. EATON, and J. E. CHRISTIAN

The measurement and classification of odors has been greatly handicapped by the lack of an objective instrument for the measurement of odor. This paper describes experiments in which the detection of odorous material in low concentrations in an air stream was made by measuring the change in surface potential of an adsorbing surface in contact with the air stream. Various solid and liquid surfaces were used including water. The effect of the presence of water vapor in the air stream on the changes in the surface potential of various surfaces was studied as well as the effect of increasing the temperature of the adsorbing surface. These tests indicate the possible use of surface phenomena for the detection of odorous material in the atmosphere.

IN AN EFFORT to gain background information for the development of an objective instrument for the measurement of odor, various surface properties of many materials have been investigated to determine whether and how they are affected by the presence of odorous materials in the vapor or gaseous state. In 1950 Tanyolac and Eaton (1) showed that the surface tension of various liquids could be used to detect the presence of, and to gain identifying information on, certain vapors from a saturated atmosphere. Following this, various odors and the deodorizing effects of certain materials were studied by the School of Pharmacy at Purdue University using surface tension measurements (2, 3). Chapman and Eaton (4) in 1955 reported that very small amounts of certain vapors in an air stream could be detected and partially identified by the measurement of changes in the surface potential of different solid surfaces. This paper outlines the continuation of this investigation at Purdue University.

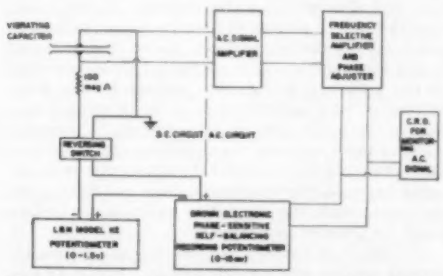

Fig. 1.—Schematic diagram of measuring circuit.

EQUIPMENT

Surface potential cannot be directly measured with the surface exposed to the atmosphere at normal pressure. However, changes in surface potential can be determined by measuring the contact potential difference between two surfaces, using as a reference one surface the potential of which does not vary. The Kelvin method for measuring contact potential differences, as modified by Zisman (5), was used in this investigation. A schematic diagram of the electrical circuit is shown in Fig. 1. The apparatus consisted of two parallel plates which formed the vibrating capacitor. The upper plate or electrode was desensitized with a coating of paraffin wax or other material so that little or no adsorption took place on the upper surface from the air stream which passed between the plates. The upper electrode was grounded and was vibrated by a small electromagnet carrying alternating current. The lower surface was always the surface upon which adsorption was

* Received March 25, 1959, from the Electrical Engineering Department, Purdue University, Lafayette, Ind.
† Present address: Electrical Engineering Department, University of Illinois, Urbana.

studied. This electrode was insulated by a teflon bushing and was resistance-capacitance coupled to the amplifier used to amplify the alternating signal from the vibrating capacitor. Semiautomatic null-balancing was provided by a phase-sensitive self-balancing recording potentiometer in series with a manual potentiometer and the vibrating capacitor. When liquid rather than solid adsorbing surfaces were used, the lower electrode of the vibrating capacitor was replaced with a shallow metal dish holding approximately 8 ml. of liquid. The polarity of the contact potential was taken as positive when the field due to the contact potential difference alone was directed upward between the two surfaces.

The air used in this research was purchased from a commercial vendor and supplied in high-pressure tanks. This was done to secure a more uniform air stream free from day-to-day variations, and also to obtain a constant pressure air supply. The air was released through a pressure regulating valve and passed through a filter composed of charcoal and anhydrous calcium chloride and calcium sulfate. From the filter, the entire air stream, or a percentage of it, could be passed through a bath of triply distilled water which was used to control the relative humidity of the stream. Three contaminant bottles could be connected to the system at one time through intervening teflon stopcocks. Contaminant vapor was injected into the air stream through displacement of the vapor in the contaminant bottle by mecury flowing in at a measured rate. A schematic air flow diagram is shown in Fig. 2.

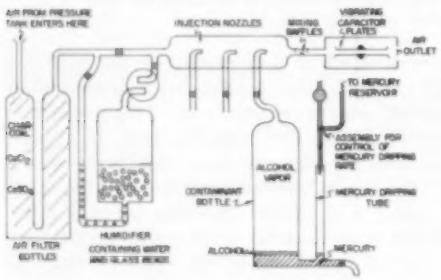

Fig. 2.—Schematic air flow diagram.

The general procedure used was to pass the air stream through a test cell containing the two plates or surfaces of the vibrating capacitor electrometer at a rate of 100 liters per hour. The surface potential of the sensitive surface was monitored for a minimum period of five minutes to make sure there was neither excessive drift nor instability in the surface potential. Contaminant vapor was then injected into the air stream at a specific rate, and the resulting change in surface potential was recorded until the surface potential again became stable, indicating equilibrium conditions for the adsorption process. At this time injection of the contaminant vapor into the air stream was stopped, and the change in surface potential was recorded until the potential again assumed a stable value. Generally, the surface potential, after an injection of contaminant vapor into the air stream, returned to or very close to the value it had prior to the contaminant vapor injection. In most cases not more than three injections of the same or different contaminant vapors were made using one particular surface, as a given surface showed a certain loss of sensitivity with repeated injections of contaminant vapor and prolonged exposure to the air stream. Surface sensitivity for a particular air stream contaminant, as used in this paper, is measured by the ratio of the change in surface potential expressed in millivolts (mv.) to the contaminant vapor concentration in the air stream expressed in parts per million by volume (p. p. m.).

EXPERIMENTATION AND DISCUSSION

Many different surfaces have been used in this investigation including gold, aluminum, charcoal, powdered zinc, powdered tellurium, mercury, and water. The most sensitive surface to many contaminants in low concentrations in the air stream was a gold plate dusted with finely powdered tellurium. With this surface the change in potential was often in the range of 20 to 80 mv. depending upon the contaminant vapor and its concentration.

As far as the detection of odorous material in an air stream was concerned, the chief disadvantage of all the solid surfaces used was their loss in sensitivity to contaminant vapor as the humidity of the air stream was increased. It was found that a dusted tellurium surface has a sensitivity of only 20% to ethyl alcohol vapor in an air stream of 30% relative humidity as compared to its sensitivity to ethyl alcohol vapor when carried by a dry air stream. This, along with the thought that if the process of olfaction is due wholly or in part to adsorption, the adsorbing surface would be most likely a liquid, was the reasoning behind the investigation of liquid adsorbing surfaces.

The outstanding advantage of a distilled water surface was that it showed no loss in sensitivity to alcohol vapor when the relative humidity of the air stream was increased to 70% and higher. This was noticeably different from all solid surfaces used and was thought to be due to the fact that the air just immediately above the water surface had a high water vapor content caused by evaporation from the surface, regardless of the humidity of the air stream. Because adsorption and desorption of water molecules was continually taking place, changes in the humidity of the air stream had a negligible effect upon the surface potential of a water surface.

Three members of the homologous alcohol series—ethyl, normal propyl, and normal butyl—were adsorbed one at a time upon a powdered tellurium surface and also upon a water surface. It was found that the overall pattern of the changes in the surface potentials for the two surfaces, shown in Figs. 3 and 4, was very similar. The change in surface potential of the tellurium surface was greater for the same amount of ethyl alcohol vapor in the air stream than that of the water surface. However, when comparisons are made between the tellurium and water surfaces, it should be noted that the adsorption of the alcohol vapor on the tellurium surface was from a dry air stream while the adsorption on the water surface was from a humid air stream.

The effect of varying the temperature of the adsorbing surface was investigated as a means of distinguishing between various air stream contaminants. This particular phase of the investigation was based on the theory that different types of adsorbates are held to the surface by different binding energies. For an adsorbed molecule to desorb from

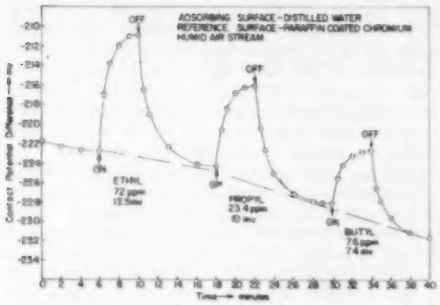

Fig. 3.—Water surface potential change due to adsorption of three different alcohol vapors.

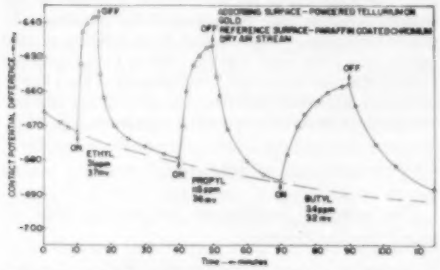

Fig. 4.—Tellurium surface potential changes due to adsorption of three different alcohol vapors.

the surface, it must be supplied with an energy equal to or greater than its binding energy, and a usual source for this escape energy is the thermal energy of the adsorbent. Thus, as the temperature of the adsorbent is increased, the greater is the chance for any given molecule to receive sufficient energy to desorb. For this reason, with constant pressure but increasing temperature, the amount of material on the surface at any one time would be expected to be less. Therefore the possibility exists that for a given surface and type of adsorbate, a unique temperature exists at which the amount adsorbed on the surface would be insufficient to cause a measurable change in the surface potential.

For the investigation of temperature dependence, it was necessary to find a surface which was sensitive to alcohol and other air stream contaminants, and one which after being heated in the presence of a pure air stream regained its original sensitivity to air stream contamination upon return to room temperature. This was necessary so that any reduced sensitivity of the surface to contaminant vapor at a higher temperature could be attributed to the increased temperature and not to a change in the surface itself due to its being heated in an air stream. To test this property of a surface, its sensitivity to a contaminant in the air stream was first measured at room temperature. Then the surface was heated in the air stream to 180° F. and allowed to cool back to room temperature. Following this, the surface was again exposed to the same contaminant at the same concentration as the original exposure and its sensitivity was again measured to determine whether it had changed. A number of solid surfaces were tested in this manner including aluminum and tellurium, but in each case their room temperature sensitivity was greatly reduced by the heating process. It was found that a surface made up of powdered wood charcoal on an aluminum plate previously heat-treated had sufficient sensitivity to alcohol vapors in the air stream, and its sensitivity at room temperature was not reduced due to its being heated in a pure air stream. This surface was adopted for this phase of the investigation.

The first tests were run using two different concentrations of ethyl alcohol vapor and one of butyl alcohol. These tests were made by first measuring the change in the surface potential at room temperature with a given concentration of the alcohol vapor in the air stream. Following this, the change in surface potential was again measured using the same vapor concentration; first, with the temperature of the adsorbing surface increased above room temperature and second, with the adsorbing surface again at room temperature. The results of a typical test of this type are shown in Fig. 5. Tests were run with the temperature of the lower plate ranging from 78 to 180° F.

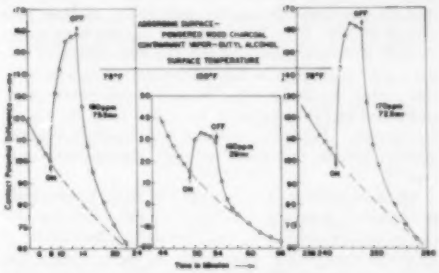

Fig. 5.—Surface potential change *vs.* time at two different temperatures.

A new surface was used for each test, which involved three equal injections of alcohol vapor into the air stream. The surfaces were all made up in the same manner using powdered wood charcoal on an aluminum plate; however, no two surfaces were alike, and their room temperature sensitivities did vary up to 20%. In order to compare the various tests, the sensitivity at the increased temperature was expressed as a percentage of the room temperature sensitivity for each individual surface used. This was done to minimize the differences in the sensitivities of the various surfaces. For calculating the per cent sensitivity at a given temperature, the room temperature sensitivity following the check at an elevated temperature was taken as 100%. For the test shown in Fig. 5 the sensitivity of the surface at 150° F. to butyl alcohol vapor was 38% of the room temperature sensitivity.

Figure 6 shows the per cent sensitivity plotted against the temperature of the adsorbing or lower surface for two concentrations of ethyl and one of butyl alcohol vapors in the air stream. The per cent sensitivity at the highest temperature reached was between 20 and 30% for both alcohol vapors. For intermediate temperatures, the differences between the per cent sensitivity for the two alcohol vapors were not appreciable and varied with the concentration of the vapor in the air stream.

Two other contaminant vapors were tested—butyl chloride and diethyl ketone. A similar reduction in the sensitivity with increased temperature was found

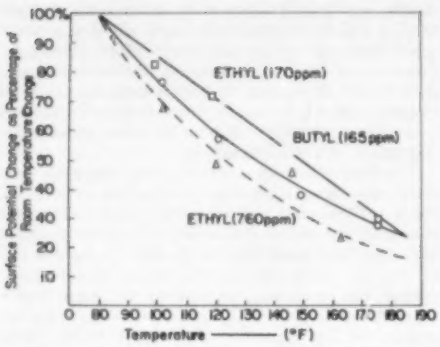

Fig. 6.—Reduction in surface potential change due to increased temperature.

using these two vapors. However, a significant difference was found in that the given concentration of butyl chloride produced no significant change in the surface potential when the adsorbing surface was heated to 165° F. Two different checks were made at 165° F.; in neither case did the butyl chloride vapor cause a change in surface potential and in each case, after the surface had cooled to room temperature, a definite sensitivity to butyl chloride vapor was found. This same pattern was established using diethyl ketone but the "cut-off" temperature was 180° F.

These tests were very limited and more work needs to be done; however, the initial results indicate the possibility of distinguishing between adsorbates of different chemical classification by determining the temperature range over which various vapors cause a measurable change in the surface potential of a stable adsorbing surface.

The tests with the alcohol vapors indicated that the highest temperature at which a change in surface potential might be detected was independent of the vapor concentration in the air stream. The fact that small increases in temperature caused a greater reduction in the per cent change in surface potential for the higher ethyl alcohol vapor concentration apparently indicates a lower binding energy for the additional molecules adsorbed because of the higher vapor concentration. Blench and Garner (6) found that the heat evolved by oxygen on charcoal was much higher for the first portions adsorbed than for latter portions. Thus, if the first portion of ethyl alcohol vapor adsorbed at either of the two concentrations used evolved the same amount of heat, it would be expected that the maximum temperature at which a change in surface potential occurred would be the same regardless of vapor concentration. This would also be necessary to distinguish between various classes of vapors by this type of test if the vapor concentrations were unknown.

Measurement of the actual amount of material adsorbed or responsible for a given change in surface potential was considered a desirable experiment in connection with this investigation. Because the amounts of adsorbed material were extremely small, the use of radioactive isotopes was considered the most feasible method for measurement of the quantity of adsorbed material. Initial experimental work was done using carbon-14 labeled ethyl alcohol. Two methods for detecting carbon-14 labeled molecules on a water surface were investigated. The first method employed a thin window carbon-14 counter placed immediately over the water surface upon which the labeled alcohol had been adsorbed. The second method involved liquid scintillation counting (7), using the water which formed the adsorbent surface as part of the liquid scintillation sample. Each method has inherent errors for which correction should be made. The major errors are due to the rapid desorption rate of the adsorbed molecules and to the solubility in water of the shorter-chain alcohols. The initial experimental results, however, definitely established the fact that the amounts of material responsible for measurable changes in the surface potential can be measured using radioactive tracer techniques. Additional work is being carried out in this area with current efforts being directed toward the simultaneous measurement of the change in surface potential and determination of the amount of adsorbed material responsible for the change.

SUMMARY

The general conclusions concerning this research project are that:

1. Measurable changes in the surface potential of many surfaces are produced by the adsorption of odorous material from the atmosphere.

2. The surface potential of a distilled-water surface is sensitive to the presence of many different organic vapors carried by an air stream, and this sensitivity is largely independent of the relative humidity of the air stream.

3. There is a possibility that vapors of different chemical classification can be distinguished by determining the adsorbing surface temperature just sufficient to prevent measurable changes in surface potential.

4. The number of adsorbed molecules causing a change in surface potential can be measured by the use of radioactive tracer techniques.

The results of these tests further set forth the possible use of surface phenomena for the detection of odorous atmospheric contamination.

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A Kinetic Study of the Hydrolysis of Methyl DL-α-Phenyl-2-piperidylacetate*

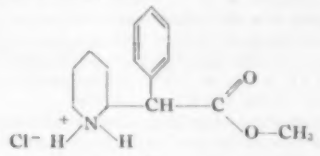
By SHELDON SIEGEL†, LEON LACHMAN‡, and LOUIS MALSPEIS

The kinetics of the hydrolysis of methyl DL-α-phenyl-2-piperidylacetate[1] in aqueous buffers were studied over the pH range 1.1 to 6.1. The hydrolysis appears to be catalyzed specifically by hydronium and hydroxyl ions. The resulting catalytic catenary afforded a pH minimum of 2.86 at 80°. The pseudo first- and second-order velocity constants for hydronium and hydroxyl ion catalysis were determined, and the temperature dependency of the reaction was studied. The influence of ionic strength on the hydronium and hydroxyl ion catalysis was investigated. The structural characteristics of the molecule contributing to the isocatalytic point are discussed.

THE CATALYTIC CATENARY of ester hydrolysis has been the subject of considerable investigation (1–3) but relatively few determinations of the catenary have been reported for cationic esters of aliphatic acids (4–8). The catenary can be calculated from the kinetic data of acid and base-catalyzed hydrolysis of esters having a permanent formal positive charge, e. g., a quaternary nitrogen (5, 6, 9–12). However, if the presence of the positive center in the molecule depends upon the hydrogen ion concentration of the solution, as occurs with substituents which are primary, secondary, and tertiary amines, the catenary cannot be determined from the second-order specific velocity constants due to hydronium ion and hydroxyl ion catalysis which are cited in the literature, since the former constant applies to hydronium ion attack on the protonated ester whereas the latter constant applies to the reaction of hydroxyl ion with the nonionic ester (13, 14). Consequently, little information is available regarding the nature of the hydrolytic catenary of a cationic ester.

The isocatalytic point of the catalytic catenary for a reaction specifically catalyzed both by hydronium and hydroxyl ions corresponds to that hydronium ion concentration at which the partial velocities due to hydronium and hydroxyl ion catalysis are equal (1). It is a composite function of the entropies and heats of activation of the hydronium and hydroxyl ion catalyses and is influenced by both steric and electronic effects of substituent groups on the substrate. Moreover, at the isocatalytic point overall reaction velocity is minimal and stability of the substrate is maximal.

The present paper reports a preliminary kinetic study of the hydrolysis of the central nervous system stimulant, methyl DL-α-phenyl-2-piperidylacetate hydrochloride (I).



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The pKa of this ester is approximately 8.8, and therefore, it is fully protonated in a solution whose pH is below 6.8. This study deals with the hydroxyl and hydronium ion—catalyzed hydrolysis in the pH range of 1.1–6.2 in order to estimate the apparent isocatalytic point of the hydrolytic catenary. Another objective of this investigation is to compare the apparent isocatalytic points, frequency factors, and energies of activation of other cationic esters with the values found for methyl DL-α-2-piperidylacetate hydrochloride.

EXPERIMENTAL

Reagents.—Methyl DL-α-phenyl-2-piperidylacetate hydrochloride, Ciba, recrystallized from ethanol and dried in vacuum, m. p. 204.5–205.5° (lit. (15) 204–208°). The reagents used as buffers and in the analytical procedures were of analytical grade. Potassium hydrogen phthalate, potassium dihydrogen phosphate, and disodium hydrogen phosphate were dried at 110° for two hours prior to use.

Eastman Kodak Company white label cyclohexane was used without further purification.

pH and Buffers Solutions.—The pH values reported are all "apparent" values inasmuch as they are subject to liquid-junction potential error. A Beckman model G pH meter provided with a fiber type saturated calomel electrode and a "General Purpose" glass electrode was used to determine the pH of samples at room temperature. Because the temperature compensation of this instrument does

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† American Foundation for Pharmaceutical Education Fellow 1955–1957. Present Address: Merck & Co., Rahway, N. J.

‡ Pharmacy Research and Development Division, Ciba Pharmaceutical Products Inc., Summit, N. J.

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not extend beyond 40°, the Beckman model H-2 pH meter was used to measure "apparent" pH values at elevated temperatures. The pH meters were standardized in the conventional manner with standard phthalate (pH 4.01 at 25°) and standard phosphate (pH 7.00 at 25°) buffers. All standardizations were carried out at the temperature of the solution being measured. The pH values of the standard buffers at elevated temperatures were those recorded by Britton (16). The pH values at elevated temperatures appear to be reproducible within ±0.02 pH unit. Temperature correction at elevated temperatures are in general accord with those of Bates (17). The concentration of the buffer used was determined by its capacity to maintain a constant hydrogen ion activity throughout the reaction. The pH of the hydrolysis mixture was determined before and after equilibration at bath temperature as well as subsequent to each kinetic run.

The buffers used in this study are listed in Table I. The ionic strength of the final solutions employed in the kinetic runs recorded in Table I were not corrected for expansion of solvent volume at elevated temperatures. They were calculated from the expression $\mu = {}^1/_2 Cz^2$ in which μ is the ionic strength, C is the concentration in moles per liter, and z is the ionic charge: pH 1.1, $\mu = 0.106$; pH 1.3, $\mu = 0.106$; pH 2.0, $\mu = 0.106$; pH 2.8, $\mu = 0.056$; pH 3.2, $\mu = 0.056$; pH 4.2, $\mu = 0.107$; pH 5.1, $\mu = 0.200$; pH 6.1, $\mu = 0.569$.

Temperature Control.—The thermostats employed were maintained at 50, 65, 80, and 95°. The 50 and 65° baths were capable of maintaining a constant temperature of about ±0.02°, the 80° bath maintained a constant temperature of about ±0.03°, while the 95° bath was capable of no better than ± 0.06°. The bath temperatures were determined with a thermometer certified by the National Bureau of Standards.

ANALYTICAL METHODS

Iron (III) Hydroxamic Acid Colorimetric Method.—For the purpose of the present study, it was desirable to develop an analytical technique which would permit the differentiation of the undegraded methyl DL-α-phenyl-2-piperidylacetate from its degradation products. The assay procedure generally used throughout this paper was an adaptation of the reaction of carboxylic esters and hydroxylamine.

Essentially, this method of assay is based on the conversion of the ester into alkali salts of hydroxamic acids. This is subsequently converted to the free hydroxamic acid by acidification and reacted with trivalent iron to form a red-colored complex. The colored complex shows an absorption spectrum in the visible range and the intensity of the color is related to the concentration of the ester.

The absorption characteristics of the iron hydroxamic acid complex of the ester are presented in Fig. 1. It can be seen from this figure that the extinction of the blank falls off quite sharply in the range of optimum absorption for the ferric hydroxamic acid complex of methyl DL-α-phenyl-2-piperidylacetate. The complex exhibits a broad maximum at 500 mμ. However, at this wave length the blank solution also shows a significant absorption, while at a wave-length from 520–540 mμ, the absorption of the blank is substantially reduced. Since at a wave-length from 520–540 mμ the complex still exhibits absorption close to maximal, it was decided to use a wave-length of 530 mμ in this study. Furthermore, by employing this higher wave length for the assay, it was possible to use higher concentrations of ferric chloride to form the colored complex. Through the use of these higher concentrations of ferric chloride the stability of the colored complex formed is increased and thereby more suitable.

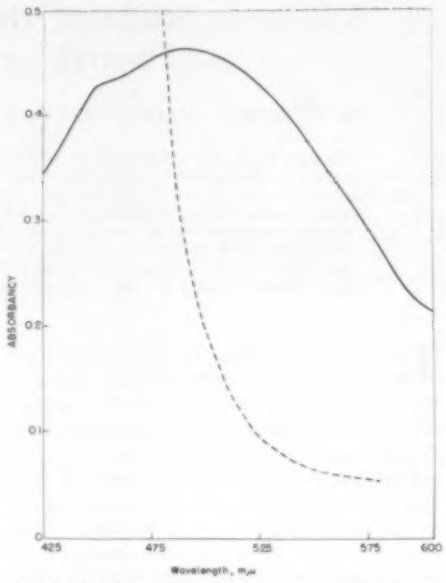


Fig. 1.—Ultraviolet absorption spectra of: ———, methyl DL-α-phenyl-2-piperidylacethydroxamic acid-ferric ion complex; - - - - -, ferric chloride blank solution.

The Beckman model DU spectrophotometer was employed to measure the absorbance of the solutions.

It was further found that the rate of reaction of ester and hydroxylamine as well as the final color intensity of the complex were pH dependent. Hestrin (18) reported that the color intensity was essentially independent of pH between pH values of 1.0 and 1.4. The final pH of the solution of the colored complex was 0.55. Attempts to decrease the hydrogen ion concentration resulted in a precipitation of ferric hydroxide due to the high ferric ion content.

Extraction Method.—The ultraviolet spectra of the ester in cyclohexane are described in Fig. 2. The cyclohexane solutions are extracts of the hydrolysis mixture which had been made alkaline at the time of extraction. Spectrum A represents the unhydrolyzed ester while spectrum B applies to the degradation which has proceeded beyond one half-life. These results suggested that the hydrolysis could be followed by determining the decrease in extinction at 260 mμ. It was established that the sodium salt of methyl DL-α-phenyl-2-piperidyl-

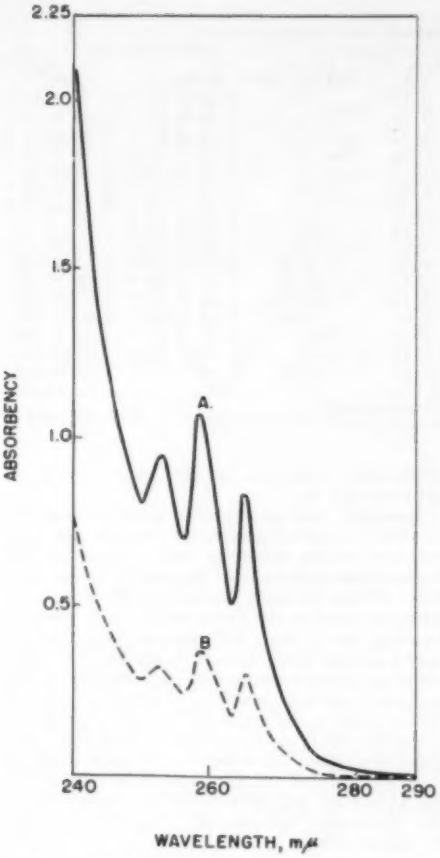


Fig. 2.—A plot of the ultraviolet absorption spectra of methyl DL-α-phenyl-2-piperidylacetate in cyclohexane. Curve A represents the absorption spectrum for the undegraded ester, while Curve B denotes the change after hydrolytic degradation.

acetate does not exhibit any significant solubility in cyclohexane as evidenced by a lack of extinction at 260 mμ.

The procedure adopted involved placing a 2.00-ml. sample of the hydrolysis mixture which had an initial known concentration of approximately 0.008 *M* ester in a ground-glass-stoppered tube and pipetting onto this sample exactly 5.00 ml. of cyclohexane. One ml. of 0.50 *M* sodium hydroxide was then added, the aqueous solution extracted by thoroughly shaking the tube, and the tube was then centrifuged for several minutes. A portion of the cyclohexane phase was then withdrawn and the absorbance determined at 260 mμ, using the smallest slit width possible, 0.7–0.8 mm. Figure 3 describes the Beer's law relationship when dilutions were performed on the aqueous solution of the ester and each solution was extracted according to the described procedure.

Excellent correlation was found between the results obtained for the colorimetric assay method and the extraction procedure. In this study, results

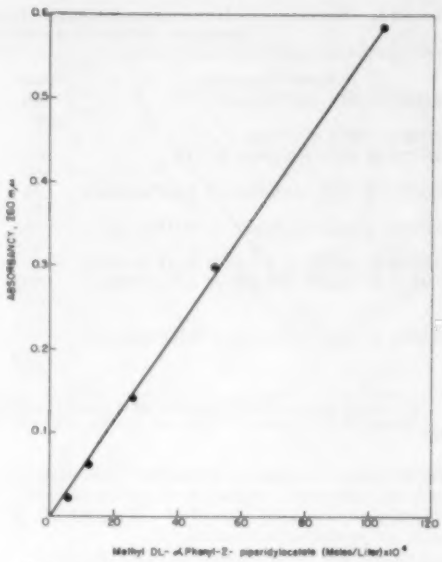


Fig. 3.—Relationship between methyl DL-α-phenyl-2-piperidylacetate concentration and absorbance in cyclohexane.

obtained by the extraction method are noted, and if no mention is made, the colorimetric assay procedure was used.

Kinetic Procedure.—A 300-mg. sample of recrystallized methyl DL-α-phenyl-2-piperidylacetate was quantitatively weighed and transferred to a 200-ml. volumetric flask and brought up to volume with buffer solution. The solution was then injected into U. S. P. XV type I ampuls of 10 ml. capacity, sealed, and immersed into the constant temperature bath. Based upon a study of equilibrium times, the solutions were allowed to equilibrate to the bath temperature of the study before removal of the sample corresponding to zero time. Ampuls were removed at specified intervals and the reaction quenched by immersion into a bath containing a mixture of ice and water.

The samples were analyzed by means of the iron (III) hydroxamic acid colorimetric method. Two ml. of the hydrolysis solution from the ampul was pipetted into a 10-ml. volumetric flask containing 1.0 ml. of 1.0 *M* hydroxylamine hydrochloride. One ml. of 2.0 *M* sodium hydroxide was added to each of the flasks and allowed to stand for fifteen minutes, acidified with 1.0 ml. of 2.0 *M* hydrochloric acid, mixed well, and the colored complex formed through the addition of 1.0 ml. of 3.0 *M* ferric chloride solution in 0.1 *M* hydrochloric acid. The mixtures in the flasks were again allowed to stand fifteen minutes, diluted to 10.0 ml. with distilled water, and the absorbance determined against the blank at 530 mμ in a Beckman model DU spectrophotometer. The extinction values for the sample were compared with values obtained for a standard solution containing 1.5 mg./ml. of ester.

The analytical procedure required modifications when pH 6 buffer was used. The hydrochloric acid solution was not of sufficient strength to lower the

TABLE I.—VARIATION OF HYDROLYSIS RATES OF METHYL DL-α-PHENYL-2-PIPERIDYLACETATE WITH APPARENT HYDRONIUM AND HYDROXYL ION CONCENTRATION

Buffer Composition	Temp.	Apparent pH[a]	Apparent $[OH^-] \times 10^{9}$[b]	k_1, min.$^{-1}$	k^1, L. mole^{-1} min.$^{-1}$
0.0946 *M* HCl + 0.0054 *M* KCl	95	1.12	...	5.82×10^{-4}	7.68×10^{-3}
	80	1.10	...	2.31×10^{-4}	2.91×10^{-3}
0.0597 *M* HCl + 0.0432 *M* KCl	80	1.32	...	1.23×10^{-4}	2.57×10^{-3}
0.0119 *M* HCl + 0.0881 *M* KCl	95	2.02	...	7.36×10^{-5}	7.70×10^{-3}
	80	2.00	...	3.17×10^{-5}	3.17×10^{-3}
0.0330 *M* HCl + 0.050 *M* KHPhthalate	95	2.81	...	3.78×10^{-5}	2.44×10^{-3}
	80	2.76	...	1.16×10^{-5}	6.66×10^{-3}
0.0204 *M* HCl + 0.050 *M* KHPhthalate	95	3.22	...	6.33×10^{-5}	...
	80	3.16	...	1.85×10^{-5}	...
0.0040 *M* NaOH + 0.100 *M* KHPhthalate	80	4.16	3.70	8.02×10^{-5}	2.17×10^{4}
0.0473 *M* NaOH + 0.100 *M* KHPhthalate	80	5.17	37.9	7.79×10^{-4}	2.06×10^{4}
	65	5.11	16.2	1.41×10^{-4}	8.71×10^{3}
	50	5.07	6.45	2.52×10^{-5}	3.90×10^{3}
0.1816 *M* NaOH + 0.200 *M* KHPhthalate	80	6.13	345	5.13×10^{-3}	1.49×10^{4}
	65	6.11	162	1.14×10^{-3}	7.02×10^{3}
	50	6.07	64.5	1.93×10^{-4}	2.98×10^{3}

[a] pH values measured with glass calomel electrodes at the temperatures cited.
[b] Calculated from apparent hydronium ion concentrations using pKw values 13.2603 at 50°, 12.9010 at 65°, and 12.5918 at 80°.

pH for color development without the precipitation of ferric hydroxide. When 1.0 ml. of 3.0 *M* hydrochloric acid was used instead of 1.0 ml. of 2.0 *M* acid, no precipitation resulted.

RESULTS AND DISCUSSION

Hydronium Ion and Hydroxyl Ion Catalysis.—The kinetic information on the hydrolysis of methyl DL-α-phenyl-2-piperidylacetate in solutions of unchanging hydronium ion concentration in the pH range 1.1–6.2 are summarized in Table I. At constant hydronium ion concentration, pseudo first-order kinetics always were observed and Fig. 4 illustrates typical semi-log plots of the fraction of unhydrolyzed ester as a function of time. The first-order rate constants, k_1, were obtained from slopes of the regressions obtained by plotting 2.303 log (100/100 − x) against time in minutes, where 100 − x represents the percentage of unhydrolyzed ester at time t. The estimated reproducibility of the first-order constants is within about 4% in the pH ranges 1–2 and 4–6, but only about 10% in the pH interval 2–4.

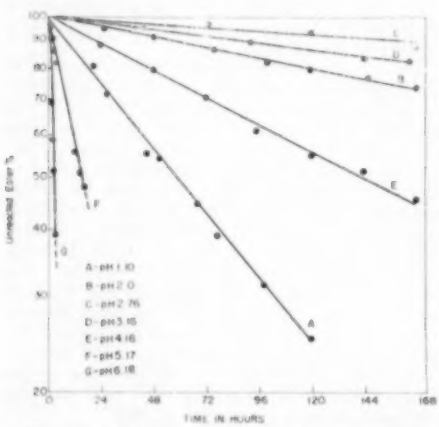


Fig. 4.—Typical pseudo first-order rate plots for the hydrolysis of methyl DL-α-phenyl-2-piperidylacetate at 80° at several pH levels.

Generally, the second-order rate constant for catalysis by a given species may be obtained from the ratio of the first-order rate constant to the thermodynamic activity of the species in the solution. However, most of the second-order rate constants cited in the literature for hydronium or hydroxyl ion catalysis were derived from the concentrations of hydronium or hydroxyl ions rather than the thermodynamic activities because of the analytical methods employed. Inasmuch as the present study is concerned with the pH dependency of the hydrolysis and the rates were determined at a given hydronium ion activity, the value obtained from the ratio of the first-order rate constant to the hydronium ion activity will be referred to as the "apparent" second-order rate constant and designated as $k^1_{H^+}$.

$$k^1_{H^+} = \frac{k_1}{A_{H_3O^+}}$$

In the pH range 1.1 to 2.0, the first-order rate constants appear to be a linear function of the hydrogen ion activity and the "apparent" second-order rate constant (Table II) was obtained from the slope of the line of a plot of $-\log k_1$ *versus* the pH using the least squares method (Fig. 5).

An added uncertainty is involved in the estimation of the second-order rate constant for the hydroxyl ion-catalyzed reaction since the hydroxyl

TABLE II.—ESTIMATED APPARENT SECOND-ORDER RATE CONSTANTS, ENERGY AND ENTROPY OF ACTIVATION FOR THE HYDRONIUM AND HYDROXYL ION-CATALYZED HYDROLYSIS OF METHYL DL-α-PHENYL-2-PIPERIDYLACETATE HYDROCHLORIDE

	H_3O^+	OH^-
$k^1$80°, L. mole^{-1} min.$^{-1}$	1.479×10^{4}	2.871×10^{-2}
Ea	15,985 (±735)	12,350 (±210)
log *A*, sec.$^{-1}$	5.79	10.15
$\Delta S^{\ddagger}$25°, cal./deg.	−34.06	−14.04

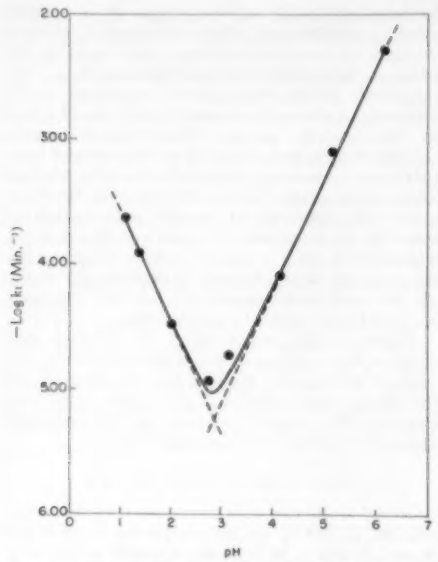


Fig. 5.—pH dependency of the hydrolysis of methyl DL-α-phenyl-2-piperidylacetate at 80°.

ion activity must be calculated from the measured pH. In addition, the "apparent" second-order rate constant, $k^1_{OH^-}$ calculated from the expression $k^1_{OH^-} = k_1 A_{H_3O^+}/Kw$ is uncertain inasmuch as an accurate value of the ion product of water, Kw, at the temperatures above 60° and ionic strength of the hydrolysis mixture is not available. Inverse linear dependence of rate on the hydronium ion activity occurs in the pH interval 4–6 (Fig. 5). The value of $k^1_{OH^-}$ was calculated from the slope of the line and is cited in Table II.

As noted by Walker and Owens (19), second-order rate constants calculated from the hydroxyl ion activity are inaccurate to at least ±5% due to a corresponding uncertainty of the pH within ±0.02 units.

The limitations that we have applied to the kinetic constants presented in this paper undoubtedly apply as well to the velocity constants reported in the literature (20) and presented in Table III.

Temperature Dependency.—The activation parameters for the hydronium and hydroxyl ion catalysis are listed in Table II. Typical Arrhenius plots are shown in Fig. 6.

The second-order hydroxyl ion rate constants were calculated as $k_1 A_{H_3O^+}/Kw$ and as noted above are approximate as Kw is uncertain. The Kw values were calculated from the Harned and Owens (21) equation

$$\log Kw = \frac{6013.79}{T} - 23.6521 \log T + 64.7013$$

However, no correction in Kw was applied for the ionic strength of the solution.

Because of the uncertainty in the activation energies estimated from the second-order rate constants, the first-order rate constants were also used to determine Ea. The energy of activation, Ea, for the

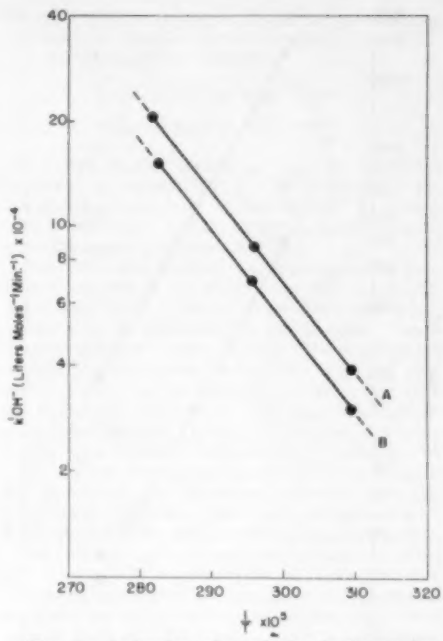


Fig. 6.—Arrhenius plot of the hydroxyl ion-catalyzed hydrolysis of methyl-DL-α-phenyl-2-piperidylacetate. A = pH 5.0, B = pH 6.0. Logarithm of the second-order rate constant against the reciprocal of the absolute temperature.

acid-catalyzed hydrolysis was found to be 15185 ± 725 cal. and the value for the base-catalyzed reaction was determined to be 25370 ± 570 cal. These values were obtained from the plots in Fig. 7. The activation energy obtained from the first-order rate constants of the hydroxyl ion-catalyzed reaction included the heat of ionization of water.

Absence of General Acid-Base Catalysis.—Bell (22) concluded from an examination of the data presented by Dawson (1) that the evidence for the general acid catalysis of ester hydrolysis is doubtful. The general acids present in the buffer systems used in this investigation are phthalic acid and the biphthalate ion (which is also a general base). To test the catalytic activity of phthalic acid, the hydrolytic rates at 95° were determined for solutions of the ester in 0.1 *M* HCl (0.0946 *M* HCl, 0.0054 *M* KCl) to which there had been added in different experiments 0.005 *M*, 0.01 *M*, 0.02 *M*, and 0.03 *M* phthalic acid. Within the experimental error, the second-order rate constants in these determinations were identical and corresponded to the rate found for hydronium ion catalysis. In another series of experiments utilizing the extraction method to follow the hydrolysis, the second-order rate constants at 95° were determined to be identical within the experimental error for solutions of the ester in 0.1 *M* HCl (0.0946 *M* HCl, 0.0054 *M* KCl) to which there had been added 0.05 *M*, 0.10 *M*, 0.20 *M*, 0.40 *M*, and 1.0 *M* acetic acid, respectively. Consequently, there is no evidence for catalysis by an undissociated acid.

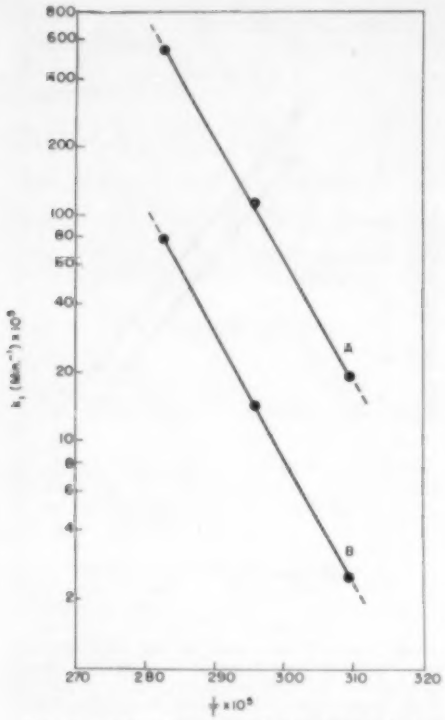


Fig. 7.—Arrhenius plot of the hydroxyl ion-catalyzed hydrolysis. A = pH 5.0, B = pH 6.0. Lygarithm of the pseudo first-order rate constant against the reciprocal of the absolute temperature.

Within the past year, substantial evidence has accrued for general base catalysis in the hydrolysis of certain esters (7, 23–25). Inasmuch as the pK_{a_2} of phthalic acid is 5.51, appreciable amounts of the general bases biphthalate ion and phthalate ion are present in the buffers at pH 5 and 6. Hydrolysis of the ester (0.0056 *M*) in a buffer containing potassium biphthalate (0.0092 *M*), potassium sodium phthalate (0.0908 *M*), and sufficient potassium chloride to adjust the ionic strength to 0.8 afforded an initial second-order velocity constant (followed only to 70% hydrolysis after which the pH of the solution increased) which was not changed when the buffer concentration was doubled at constant ionic strength. Hence, it appears that the hydrolysis is catalyzed by neither biphthalate ion nor phthalate ion.

The evidence presented that the hydrolysis is not a general acid-base catalyzed process is tempered by the experimental error, and it is necessary to conclude that any contribution to the hydrolytic rate due to general acid-base catalysis is negligible relative to the respective catalysis by hydronium ion and hydroxyl ion. Thus, the hydrolysis appears to be specifically hydronium and hydroxyl ion-catalyzed.

Ionic Strength Effect.—From the Bronsted-Bjerrum equation (26) primary kinetic salt effects are expected for the specific hydronium ion and hydroxyl ion-catalyzed hydrolysis of a cationic ester; a positive salt effect occurring in the hydronium ion-catalyzed reaction and a negative salt effect in the hydroxyl ion-catalyzed reaction. The magnitude of the effect should be related to the proximity of the positive center to the site of attack by the catalytic species. Characteristic primary salt effects have been observed in the acid and base-catalyzed hydrolysis of carbethoxymethyltriethylammonium halide (11, 12, 27), and in the base-catalyzed hydrolysis of methyl pyrrolidylacetylsalicylate hydrochloride (7) and acetylcholine (6). These effects do not appear to have been noted in the acid and base-catalyzed hydrolysis of atropine (8, 28) and acetylcholine (9) and in the base-catalyzed hydrolysis of procaine (29).

Neglecting the minor effect of changing ionic strength in a solution of unchanging dielectric constant on the reaction between the attacking ion and the dipolar ester functional group, the major ionic strength effect should conform to the expression applied to reaction between ionic species

$$\log k = \log k_0' + 1.02\, z_A z_B \sqrt{\mu}$$

where k_0' is the rate constant in an infinitely dilute solution, z_A and z_B are the charges on the reactants *A* and *B*, and μ is the ionic strength of the solution (26). This expression is applicable to very dilute solutions, $\mu = 0$ to 0.4, and marked deviations from the relationship have been observed with increasing ionic strength. Nevertheless, a plot of the logarithm of the second-order velocity constants against the square root of the ionic strength should yield information regarding the primary kinetic salt effect.

Figure 8 shows a plot of log $k^1_{H^+}$ as a function of $\sqrt{\mu}$ and illustrates the positive primary salt effect observed in the hydronium ion-catalyzed reaction. Hydrolysis of the ester (0.0093 *M*) in 0.1 *M* HCl and made up to final ionic strengths with sodium chloride was followed at 95° using the extraction method of analysis. No correction of the ionic strength was applied due to expansion of the solvent. Second-order velocity constants were calculated from the first-order constants using the measured hydrogen ion activities. At $\mu = 0.50$, $k^1_{H^+} = 9.80 \times 10^{-3}$ L. moles^{-1} min.$^{-1}$, $\mu = 1.00$ $k^1_{H^+} = 11.00 \times 10^{-3}$ L. moles^{-1} min.$^{-1}$, and at $\mu = 2.00$, $k^1_{H^+} = 13.08 \times 10^{-3}$ L. moles^{-1} min.$^{-1}$. Linear dependence of the plot of log $k^1_{H^+}$ against $\sqrt{\mu}$ is found, however, the slope of the curve is 0.47 rather than the value of unity indicated in above equation. The observed ionic strength effect for the acid-catalyzed reaction appears to be relatively small. Undoubtedly, the results obtained are only valid within the experimentally determined ionic

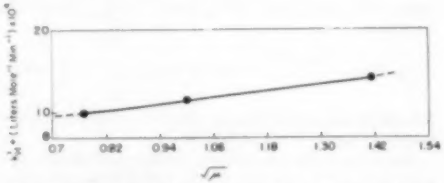


Fig. 8—Influence of ionic strength on the velocity of hydronium ion-catalyzed reaction.

strength range and may not be extrapolated to lower ionic strengths where the slope is expected to increase.

A comparable negative salt effect was observed for the hydroxyl ion-catalyzed reaction. Employing the extraction method of analysis, first-order velocity constants at 95° for the hydrolysis of the ester (0.0093 *M*) in buffers containing 0.2624 *M* NaH_2PO_4, 0.1504 *M* Na_2HPO_4 adjusted to final ionic strengths with sodium chloride were experimentally determined. Second-order rate constants were calculated as previously described. At $\mu = 0.50$, $k^1_{OH} = 3.54 \times 10^4$ L. mole^{-1} min.$^{-1}$, $\mu = 1.00$, $k^1_{OH} = 3.30 \times 10^4$ L. mole^{-1} min^{-1}, and at $\mu = 3.00$, $k^1_{OH} = 2.77 \times 10^4$ L. mole^{-1} min^{-1}. A plot of log k^1_{OH} against $\sqrt{\mu}$ proved to be linear (Fig. 9) and illustrates a negative salt effect. As before, the slope of the plot, 0.307, deviates from unity. Because of the demonstrated primary salt effects, the rate constants determined in this study apply only to the vicinity of the experimental ionic strengths.

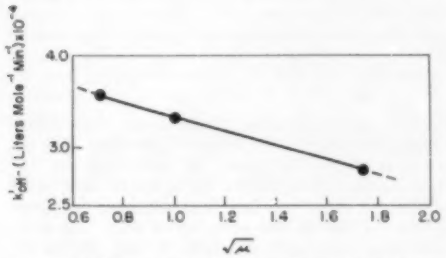


Fig. 9.—Influence of ionic strength on the velocity of the hydroxyl ion-catalyzed reaction.

The Hydrolytic Catenary.—The first-order rate of the hydrolytic reaction in aqueous solution which is specifically catalyzed by the hydronium and hydroxyl ions is given by $k_1 = k_{H_2O}[H_2O] + k_{H^+}[H_3O^+] + k_{OH^-}(Kw)/[H_3O^+]$ (1). In the present paper k_{H^+} and k_{OH^-} are replaced by $k^1_{H^+}$ and $k^1_{OH^-}$. When the rate of change of k_1 with a change in $[H_3O^+]$ is minimal, $dk_1/d[H_3O^+] = 0$ and $k^1_{H^+} = Kw\ k^1_{OH^-}/[H_3O^+]^2$; the hydronium ion concentration at this minimal overall velocity is noted as $[H_3O^+]_0$ and corresponds to the isocatalytic point since $k^1_{H^+}[H_3O^+]_0 = Kw\ k^1_{OH^-}/[H_3O^+]_0$. The minimal velocity, v_0 at this isocatalytic point is given by $v_0 = 2\,k^1_{H^+}[H_3O^+]_0$ or $v_0 = 2\sqrt{k^1_{H_3O^+}\,k^1_{OH^-}\,Kw}$ if the contribution of $k_{H_2O}[H_2O]$ is neglected.

The experimentally determined pH dependency of the hydrolysis at 80° is presented as a plot of $-\log k_1$ against pH in Fig. 5. The dominant feature of the curve is that the pH_0 appears to be 2.86 where the corresponding $k_1 = 9.33 \times 10^{-4}$. The pH_0 calculated from the second-order velocity constants estimated from the slopes of the linear segments of the pH dependency curve, $k^1_{H^+} = 2.87 \times 10^{-3}$ L. mole^{-1} min.$^{-1}$ and $k^1_{OH^-} = 1.48 \times 10^4$ L. mole^{-1} min.$^{-1}$, is 2.94 and the corresponding value of k_1 is calculated to be 6.59×10^{-4}. This low isocatalytic point is not unexpected qualitatively as a shift to a lower isocatalytic point parallels a large $k^1_{OH^-}/k^1_{H^+}$, which in the case of a cationic ester is considerable for $k^1_{OH^-}$ is increased as a result of the electrostatic attraction of the molecule for the hydroxyl ion whereas $k^1_{H^+}$ is decreased through the electrostatic repulsion for the proton.

From the Arrhenius equations

$$k^1_{H^+} = 3.68 \times 10^7 e^{-15,900/RT}$$

$$k^1_{OH^-} = 8.57 \times 10^{11} e^{-12,350/RT}$$

the values of $k^1_{H^+}$ and $k^1_{OH^-}$ may be calculated for any given temperature. At 25°, for example, the calculated values, $k^1_{H^+} = 7.02 \times 10^{-6}$ L. mole^{-1} min.$^{-1}$ and $k^1_{OH^-} = 7.59 \times 10^9$ L. mole^{-1} min.$^{-1}$ yield the isocatalytic point 3.48.

The difference between the experimentally estimated velocity of the reaction at the isocatalytic point and that calculated from $k^1_{H^+}$ and $k^1_{OH^-}$ values may be attributed to the so-called "spontaneous reaction" or $k_{H_2O}[H_2O]$. The contribution of $k_{H_2O}[H_2O]$ estimated in this way is 2.74×10^{-4} min.$^{-1}$ which is a substantially smaller value than the value 4.67×10^{-4} min.$^{-1}$ for $k_{H_2O}[H_2O]$ determined by Garrett (7) for the protonated ester methyl pyrrolidylacetylsalicylate hydrochloride. In addition, this exceedingly small difference between the estimated and calculated minimal velocities supports the postulate that there is no general acid-base catalysis in the vicinity of the isocatalytic point.

For comparison purposes, the isocatalytic point of the hydrolysis of esters reported in the literature can be calculated from the specific velocity constants cited providing the ionic charge on the ester is the same in both determinations. Unfortunately, most second-order constants for the hydroxyl ion-catalyzed reaction which have been determined apply to the nonprotonated ester. The isocatalytic points of many nonionic esters are in the range 5.3 to 5.6 (2). The value for ethyl aminoacetate is 3.8 at 20° (4) and hence it is apparent that the value of methyl DL-α-phenyl-2-piperidylacetate is of the proper order of magnitude. For comparison, the isocatalytic points of the cationic esters atropine and acetylcholine are 3.24 at 100°(8) and 3.73 at 25° (9), respectively. These values indicate that these compounds contain structural features which increase the ratio $k^1_{OH^-}/k^1_{H^+}$.

STRUCTURAL CONSIDERATIONS

The discussion presented above is consistent with Bender's mechanisms of ester hydrolysis (30) applied to a cationic ester. The recent studies of concurrent oxygen exchange and acid and alkaline hydrolysis of Bender (31) support the concept that a similar intermediate is involved in both the acid and base-catalyzed reactions. Inasmuch as substituent groups will influence this intermediate, the effects of these groups may be compared for the hydronium ion and hydroxyl ion-catalyzed reactions. Although methyl DL-α-phenyl-2-piperidylacetate may be viewed as a substituted methyl propionate or as an ester of heptanoic acid, it will be considered a substituted acetate for comparison purposes.

The low solubilities of nonionic esters in water has precluded kinetic studies of hydrolysis in this solvent, and it is necessary to extrapolate information from ethanol-water and acetone-water systems. In Table III, values of the energy of activation, *Ea*, for the hydronium ion-catalyzed hydrolysis of methyl acetate are only very slightly higher in water than in 60% acetone and *A* increases almost

TABLE III.—ARRHENIUS CONSTANTS[a] FOR THE HYDRONIUM AND HYDROXYL ION-CATALYZED HYDROLYSIS OF SOME ESTERS[b]

	Log A	Ea	Log A	Ea	Reference
Methyl DL-α-phenyl-2 piperidylacetate	5.79	15,980	10.16	12,350	([h])
Atropine[g]	5.41	17,200	10.32	12,700	(8)
Procaine[g]	...	...	8.87	12,000	(29)
Scopolamine methylbromide	...	...	8.61	11,800	(37)
Acetylcholine	7.67	16,570	9.00	12,180	(9)
Carbethoxymethyltriethylammonium chloride	...	...	10.82	12,700	(11)
Methyl acetate	7.64[c]	16,250	7.57[c]	11,500	(39)
	8.59	16,920	...	...	...
Ethyl acetate	...	...	8.20[d]	14,200	(38)
	...	...	8.6[d]	14,700	(36)
	7.53[e]	16,200	5.9[e]	9,800	(33)
Ethyl propionate	...	...	6.78	10,720	(32)
	...	...	8.34[f]	14,500	(32)
Ethyl phenylacetate	7.08[e]	16,200	...	...	(33)
	6.97[e]	16,100	7.34[d]	14,100	(35)
Ethyl diethylacetate	8.08[e]	19,100	6.4	14,300	(33)
			7.96[d]	17,400	(40)
			7.8[d]	17,300	(36)

[a] Log k = log A − $Ea/2.303RT$ as estimated from rate constants where k is L. mole^{-1} sec.$^{-1}$.
[b] Solvent is water unless otherwise noted.
[c] 60% acetone.
[d] 85% ethanol.
[e] 70% acetone.
[f] 76.5% ethanol.
[g] Arrhenius parameters are for the protonated ester.
[h] This investigation.

by one factor of ten. It appears likely, therefore, that the Arrhenius parameters determined in 60 to 70% acetone for the acid catalysis are similar to those in water. However, considerable differences in the parameters have been observed for the base-catalyzed reaction. The data of Tommila and Hietala (32) for the hydroxyl ion-catalyzed hydrolysis of ethyl propionate in water and 76.5% ethanol (Table III) indicate that the energy of activation is approximately 3.5 Kcal./mole greater in the latter solvent and that A has increased by a factor of 15. Moreover, Davies and Evans (33) have compared values of Ea for alkaline hydrolysis in 70% acetone and 85% ethanol for the saponification of normal esters noting that the values are roughly 3.5 Kcal./mole greater in the hydroalcoholic solution. Therefore, while only gross comparisons are permitted, Ea values in water and acetone-water solutions are similar, whereas Ea values determined in 85% ethanol must be reduced by approximately 3.5 Kcal./mole for comparison purposes.

The effect of the α-phenyl substituent on acid and base-catalyzed ester hydrolysis has been extensively investigated (34, 35). In the acid-catalyzed hydrolysis of ethyl phenylacetate compared to ethyl acetate, Log A is reduced by 0.5 and Ea is unchanged. This effect has been attributed to the steric effect of the phenyl group, the negative inductive ($-I$) effect being negligible. The effect of the α-phenyl group on the base-catalyzed reaction is to reduce Ea by 600 cal. and Log A by 1.3; these results being due to the $-I$ and steric effects. Effects of similar magnitude should be manifested by the α-phenyl group in methyl DL-α-phenyl-2-piperidylacetate. Thus the observed changes in the frequency factor cannot be significantly attributed to the α-phenyl group. Apparently, a large contribution to the changes in frequency factor is due to the piperidine substituent.

The union of the piperidine ring to the acetic acid residue is at the 2 position. The effect of an alkyl radical on the α-carbon of the acetic acid moiety should account for the effects other than that attributed to the nitrogen. In acid-catalyzed hydrolysis, Ea is increased by 100 to 400 cal. as a result of the $+I$ effect and Log A is decreased by about 0.1 to 0.4 due to the steric effect (33). In base-catalyzed hydrolysis, Ea may or may not be increased up to 400 cal. depending on the substituent ($+I$ effect) and in most cases a small decrease in Log A is observed, e. g., 0.4 to 0.8, which is very likely due to decreased hyperconjugation (36). An extreme case of the effects of α-alkyl-substituents on ester saponification would be represented by ethyl diethylacetate which has an unusually high activation energy (Table III). These effects are relatively minor and consequently, the major contribution to the difference in the Arrhenius parameters of methyl DL-α-phenyl-2-piperidylacetate and acetic ester are due to the protonated nitrogen.

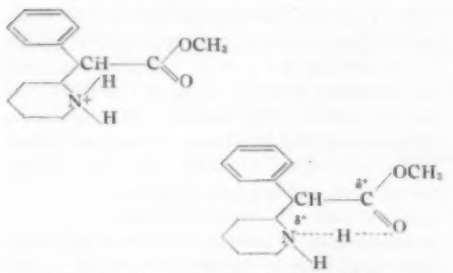
In Table III, the Arrhenius constants for hydronium ion-catalyzed hydrolysis of methyl DL-α-phenyl-2-piperidylacetate, atropine, and acetylcholine are listed. The activation energies of these compounds are similar, whereas the nonexponential factor of atropine and methyl DL-α-phenyl-2-piperidylacetate are smaller than that of acetylcholine or a nonionic ester. Atropine is an ester of a secondary alcohol in addition to possessing a cationic center somewhat remote from the ester functional group and steric effects are manifested in both the alkyl and acid moieties. Thus a comparison of atropine and methyl DL-α-phenyl-2-piperidylacetate merely points up the large effect due to the cationic center in methyl DL-α-phenyl-2-piperidylacetate. It may be noted that in acetylcholine, without a contribution of steric hindrance, the hydrolysis rate is reduced by only a small extent as compared to a nonionic ester.

The activation energies for the hydroxyl ion-catalyzed hydrolysis of the cationic esters listed in Table III are surprisingly similar to one another

considering their diverse structures. In both acetylcholine and carbethoxymethyltriethylammonium chloride, the quaternary nitrogen is closer to the center of hydronium ion attack than is the protonated nitrogen in methyl DL-α-phenyl-2-piperidylacetate with consequent greater negative inductive effects. In addition, the *Ea* values of the cationic esters appear to be consistently larger than that of nonionic esters (values extrapolated to aqueous solution). As noted by Bell (11), the greater *Ea* is the result of an abnormally high collision factor resulting from the electrostatic attraction for the hydroxyl ion by the cationic ester. It appears that the collision factor makes a major contribution to the hydrolysis rate in cationic esters while the inductive effects are minor in importance.

The frequency factors of the cationic esters are all greater than those of the nonionic esters and that of methyl DL-α-phenyl-2-piperidylacetate is seen to be similar in magnitude to those esters whose cationic center is closer to the carbonyl-carbon. It is evident that the large decrease in rate in the hydronium ion-catalyzed reaction and the increase in rate in the hydroxyl ion-catalyzed reaction of methyl DL-α-phenyl-2-piperidylacetate are accompanied by corresponding changes in the entropies rather than the energies of activation; at 25°, $\Delta S^{\ddagger}$ (20) corresponding to $k^{1}_{H^+}$ is −34.06 E.U. and to $k^{1}_{OH^-}$ is −14.04 E.U.

Conceivably, these results can be accounted for in terms of intramolecular hydrogen bonding of the protonated nitrogen to the carbonyl-oxygen.



Methyl-DL-α-phenyl-2-piperidylacetate

Inspection of a Fisher-Hirshfelder-Taylor model shows that such hydrogen bonding can readily occur. Placement of a fractional positive charge should enhance the approach of the hydroxyl ion to the carbonyl-carbon resulting in an increase in the collision factor and frequency factor. The approach of a proton to the methoxy-oxygen should be repelled by a carbonyl-carbon with enhanced electrophilic character, although the approach of the nucleophilic water molecule to the carbonyl-carbon should be facilitated. Therefore, in the pH range 2 to 3, the hydronium ion concentration is sufficiently low that electrostatic repulsion of the available protons limits the reaction velocity, but as the hydrogen ion concentration is increased, the attraction of the carbonyl-carbon for the nucleophilic water molecules results in an increased reaction velocity. Alternately, increased velocity with increasing hydronium ion concentration may be due to a loss of stability of the hydrogen bond as the proton approaches the carbonyl-oxygen.

CONCLUSIONS

1. The hydrolytic reaction appears to be catalyzed specifically by hydronium and hydroxyl ions.

2. A positive primary salt effect seems to be present for the hydronium ion-catalyzed reaction and a negative salt effect has been observed for the hydroxyl ion-catalyzed reaction.

3. The pH of maximum stability for the ester in aqueous solutions is 2.86 at 80°.

4. At a pH of 2 or less and 4 or higher, the reaction is essentially catalyzed by hydronium and hydroxyl ion, respectively.

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Veratrum Alkaloids XXXII*

The Structures of Germitetrine and Some Related Hypotensive Ester Alkaloids

By S. MORRIS KUPCHAN and C. IAN AYRES

The structures of germitetrine and several related hypotensive esters of germine have been elucidated. The chemically-related series germitetrine, desacetylgermitetrine, and neogermbudine are formulated as I, IV, and V, respectively.

ALKALOIDAL MIXTURES obtained from *Veratrum album* are employed in the treatment of hypertension. One of the most powerful hypotensive constituents of these alkaloidal mixtures is germitetrine (1–4) whose structure elucidation was recently described (5) in a brief preliminary communication. The present report details the evidence for assignment of structure I to germitetrine.

Alkaline hydrolysis of germitetrine has afforded the known alkamine germine (II) (6), two mole equivalents of acetic acid, one mole equivalent of (*l*)-2-methylbutyric acid, and one mole equivalent of the low melting isomer of 2,3-dihydroxy-2-methylbutyric acid (2, 4). Methanolysis of germitetrine was shown to yield two degradation products. The loss of one acetate grouping led to the triester desacetylgermitetrine, whereas removal of both acetate groupings resulted in conversion to the naturally-occurring germine diester, neogermbudine (4). The acetate grouping methanolyzed in the conversion of germitetrine to desacetylgermitetrine has been provisionally assigned to C_7 on the basis of molecular rotation differences and the characteristically facile methanolysis of C_7 acetates (7).

In a recent review (8), the four acyl groups of germitetrine were tentatively assigned to positions C_3, C_7, C_{15}, and C_{16} of germine. We were led to question this proposal on the basis of a preliminary structure-activity generalization which appears to exist among the hypotensive veratrum derivatives. To date, all the hypotensive naturally-occurring germine esters of known structure possess a free hydroxyl group at C_{16} (7).

An indication that the C_3, C_7, C_{15}, C_{16} tetraester structure was untenable came from the observation that germitetrine was completely stable toward sodium periodate. The proposed tetraester would have a free glycol present in its DMB residue and would therefore be readily attacked by sodium periodate. Furthermore, germitetrine was shown to possess a free C_{16} hydroxyl group in the following manner. Chromic acid titration showed the presence of only one secondary hydroxyl group. The oxidation product, germitetrone (VI) (m. p. 222–223°decompn.), on alkaline hydrolysis gave the diosphenol (VII) previously obtained from neogermitrone and 16-dehydrogermine 3,6,7,15-tetraacetate (6, 7). Thus germitetrine is a germine 3,7,15-triester in which the secondary hydroxyl group present in the DMB residue is acylated, thus accounting for the four mole equivalents of acid produced on hydrolysis.

The location of germitetrine's more easily methanolyzed acetyl residue at C_7 requires that desacetylgermitetrine be a germine 3,15-diester in which the secondary hydroxyl group of the DMB residue is acylated. The remaining structural questions were settled by studies on neogermbudine, the germine mono-MB mono-DMB which is obtained upon methanolysis of desacetylgermitetrine (see Table I). Neogermbudine consumed 1 mole equivalent of sodium periodate in agreement with assignment of the acyl residues at C_3 and C_{15}. The specific location of each substituted butyryl residue was determined by use of the reported selectivity of the sodium borohydride reduction of esters of hydroxyacids (9, 10). When neogermbudine was treated with sodium borohydride, the dihydroxybutyryl ester grouping was selectively removed and a known (11) germine mono-(*l*)-2-methylbutyrate was isolated. This compound consumed 1.1 mole equivalents of sodium periodate in agreement with the germine 15-(*l*)-2-methylbutyrate structure tentatively assigned on other grounds (7). Furthermore, the amorphous oxidation product showed infrared absorption at 3.65, 5.62, and 5.80 μ characteristic of the aldehydo-γ-lactone resulting from cleavage of the ring A glycol (6). Thus, neogermbudine is germine 3-DMB-15-MB (V), desacetylgermitetrine is germine 3-HMAB 15-MB (IV), and germitetrine is germine 3-HMAB 7-Ac 15-MB (I).

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Part XXXI in the series: S. M. Kupchan, N. S. Johnson, and S. Rajagopalan, *Tetrahedron*, in press.

CHART 1

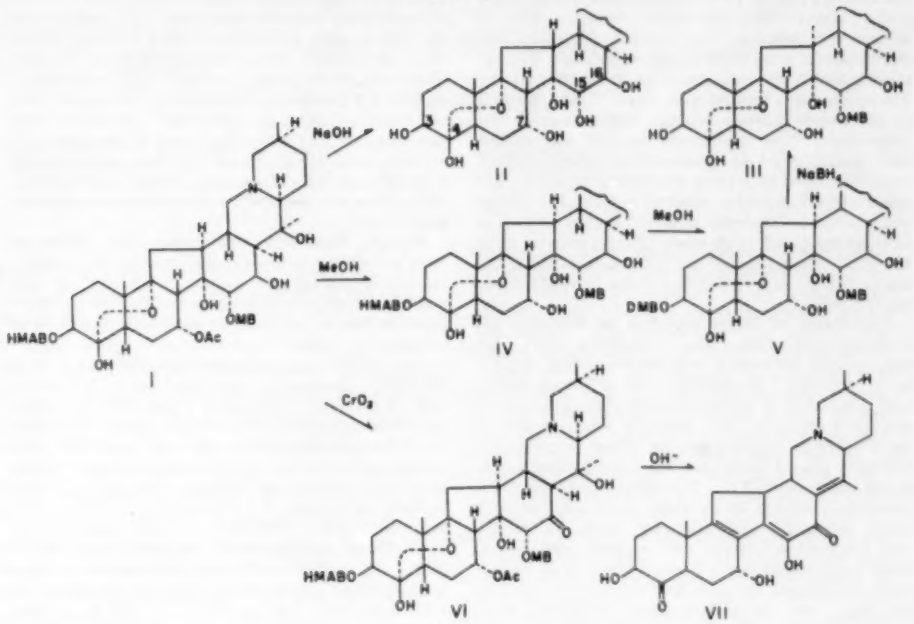

TABLE I.

Germitetrine (germine-mono Ac-mono MB-mono HMAB) ↓ NaOH Germine	⟶ MeOH[4,7]	Desacetylgermitetrine (germine-mono MB-mono HMAB)	⟶ MeOH[4] Neogermbudine (germine-mono MB-mono DMB) ↓ $NaBH_4$ Germine-15-(*l*)-2-methylbutyrate

MB = (*l*)-2-methylbutyryl. DMB = (*l*)-2,3-dihydroxy-2-methylbutyryl. HMAB = 2-hydroxy-2-methyl-3-acetoxybutyryl.

EXPERIMENTAL

Melting points are corrected for stem exposure. Values of $[\alpha]_D$ have been approximated to the nearest degree. Ultraviolet absorption spectra were determined on a model 11 MS Cary recording spectrophotometer and 95% ethanol was used as solvent. Infrared spectra were determined on a Baird model B double beam infrared recording spectrophotometer and chloroform was used as a solvent. Microanalyses were carried out by Dr. S. M. Nagy and his associates at the Massachusetts Institute of Technology on samples dried at reduced pressure at 110°.

Oxidation of Germitetrine to Germitetrone (VI).—Germitetrine (39.3 mg.), m. p. 229–231° (decompn.), in glacial acetic acid (5 cc.) was treated at room temperature with a solution of chromic anhydride in acetic acid containing 0.2% of water (5 cc., 0.06 *N*). Aliquot parts were titrated at intervals. With the aid of the appropriate blank, the rate of consumption of chromic acid was shown to be as follows: forty minutes, 0.74 of the theoretical amount of reagent required for the oxidation of one secondary hydroxyl group; one hour, 0.80 of theoretical; two hours, 0.89 of theoretical; twenty hours, 1.16 of theoretical.

Germitetrine (300 mg.), m. p. 229–231° (decompn.) in glacial acetic acid (4 cc.) was treated with 0.66 *N* chromic anhydride in 98.5% acetic acid (8 cc.). The dark brown mixture was allowed to stand at room temperature for seventy-five minutes. The reaction mixture was cooled in an ice bath and treated first with dilute sodium bisulfite to a green color and then with dilute ammonia until alkaline. The solution was extracted with chloroform and the chloroform solution was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness at reduced pressure. The residue crystallized from 95% ethanol in the form of colorless prisms (250 mg.), m. p. 220–221° (decompn.). Recrystallization of a sample for analysis from 95% ethanol afforded colorless prisms, m. p. 222–223° (decompn.); $[\alpha]_D^{25}$ −167° (c 1.08, py.).

Anal.—Calcd. for $C_{31}H_{41}O_{14}N.H_2O$: C, 60.79; H, 7.84. Found: C, 61.18; H, 7.88.

In a volatile acid determination (12) 26.00 mg. of the ketone yielded an amount of acid equivalent to 13.53 cc. of 0.007317 *N* sodium thiosulfate; calcd. for two mole equivalents of acetic acid and one mole equivalent of (*l*)-2-methylbutyric acid, 13.16 cc.

Alkaline Treatment of Germitetrone.—Germitetrone (30 mg.), m. p. 220–221° (decompn.), was

treated with methanol (3 cc.) and 50% sodium hydroxide solution (0.1 cc.) and the solution was heated under reflux for twelve minutes. The resulting red solution was treated dropwise with glacial acetic acid until a yellow solution was obtained, and then evaporated at reduced pressure. The residue was treated with water (5 cc.), brought to pH 9 with dilute ammonia, and extracted with chloroform. The chloroform solution was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness at reduced pressure. The residue was dissolved in ether (5 cc.) and the solvent was allowed to evaporate at room temperature. The light brown powdery residue (12 mg.) showed m. p. 170–180° (decompn.); $\lambda_{max.}$ 320 mμ (ε 13,000), 285 mμ (ε 7,000); $\lambda_{max.}^{0.01N\ KOH}$ 372 mμ (ε 10,000), 345 mμ (ε 8,000).

Conversion of Neogermbudine to Germine 15-(*l*)-2-methylbutyrate (III).—Neogermbudine[1] (100 mg., Ayerst McKenna and Harrison Ltd. R7593, m. p. 149–152°) in methanol (3 cc.) was treated with a solution of sodium borohydride (100 mg.) in methanol (9 cc.). The solution was allowed to stand at room temperature for fifteen hours, acidified with glacial acetic acid, and evaporated at reduced pressure at 30°. The residue was dissolved in water, cooled in an ice bath, made alkaline with dilute ammonia, and extracted with chloroform. The chloroform extract was washed with water, dried over anhydrous sodium sulfate, and evaporated at reduced pressure to dryness. The residue (72 mg.) was chromatographed on Merck acid-washed alumina (10 Gm.) and 40-cc. fractions of eluate were collected. Fractions 1, 2, and 3 eluted with chloroform, fractions 4 and 5 eluted with 1% methanol in chloroform, and fractions 6 and 7 eluted with 2% methanol in chloroform did not yield any solid material. Fraction 8, eluted with 7% methanol in chloroform, yielded a resin consisting mainly of starting material, as indicated by paper chromatographic comparison with neogermbudine. The procedure employed was that described by Levine and Fischbach (13) for "slower moving" alkaloids. Fraction 9, eluted with 10% methanol in chloroform, yielded 17 mg. of residue; fraction 10, eluted with the same solvent, gave 10 mg. of residue; fraction 11, eluted with 15% methanol in chloroform, gave 8 mg. of residue (total solid eluted: 61 mg.). Fractions 9,10, and 11 consisted largely of germine 15-(*l*)-2-methylbutyrate, as indicated by paper chromatographic comparison with an authentic sample.[2] The three fractions were, therefore, combined in chloroform and filtered through a column of Merck acid washed alumina (1 Gm.) to remove some colored impurities. Evaporation of the filtrate gave a colorless residue (35 mg.) which was crystallized from chloroform-ether to give elongated prisms (9 mg.), m. p. 225–229° (decompn.) with slight preliminary browning. A second crop of prisms (15 mg.), m. p. 216–219° (decompn.), was also obtained. The melting point of the first crop material was not depressed by admixture with the authentic sample of germine 15-(*l*)-2-methylbutyrate. The infrared spectra of the respective samples were identical.

Sodium Periodate Oxidations.—The titrations were performed as in our earlier studies (6). Germitetrine consumed no sodium periodate in nine hours. Neogermbudine consumed 0.84 mole equivalent in two hours; 0.95 in six hours, and 1.03 in twenty-four hours.

Germine 15-(*l*)-2-methylbutyrate (27.3 mg.), m. p. 225–229° (decompn.), was dissolved in 5% acetic acid (2 cc.) and water (3 cc.) and 0.08 *M* sodium periodate solution (5 cc.) were added. Titration of a 2-cc. aliquot after two and one-half hours showed an uptake of 1.07 mole equivalents. Titration of a second 2-cc. aliquot after twenty hours showed an uptake of 1.30 mole equivalents. The remainder of the solution (6 cc.) was made alkaline with dilute ammonia and extracted with chloroform. Evaporation of the chloroform left an amorphous residue which showed infrared absorption at 3.65, 5.62, and 5.80 μ. These bands have been shown to characterize the Ring A aldehydo-γ-lactone structure (6).

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[1] We thank Dr. G. S. Myers of Ayerst McKenna and Harrison Ltd. for a generous gift of neogermbudine.
[2] We thank Dr. J. Bolger of Riker Laboratories, Inc. for a comparison sample of germine 15-(*l*)-2-methylbutyrate.

Intermediary Metabolism of Ergot I*

Amino Acid Metabolism

By GUNNAR GJERSTAD

A comparative analysis of the amino acid metabolism of parasitic and saprophytic ergot is reported. Identification was effected by two-dimensional paper partition chromatography.

IN RECENT YEARS considerable interest has centered around the problem of making the ascomycete, *Claviceps purpurea* (Fries) Tul., produce the medicinally important ergot alkaloids when grown in synthetic or semisynthetic substrates (1). Occasional reports that alkaloids have been extracted from the fungus cultivated in artificial media have proved to be nonreproducible in the United States using local isolates of *Claviceps* (2).

In a previous publication (3) the influence of the addition of several chemical compounds to synthetic substrates was investigated in an attempt to test for any precursor effects. The results indicated strongly that the failure of alkaloid formation was most plausibly not due to a nutritional deficiency of the employed substrates.

Since alkaloids were not formed, it appeared of great interest to investigate if *Claviceps* would show any other metabolic differences when grown on rye as compared with the same strain raised in submerged cultures.

EXPERIMENTAL

Cultures.—The initial isolate of the employed fungus was obtained through courtesy of Eli Lilly & Co., Indianapolis, and described as rye ergot of a very high alkaloid yielding strain. The culture which has been maintained for several years on Kirchhoff's agar medium (4), was inoculated on Balbo rye Tennessee by dipping the heads into a conidio spore suspension, and in the course of fifty days, mature sclerotia developed. The same conidio culture was grown in submerged culture in the laboratory for an identical period of time.

Media.—Since we were interested in analyzing the resulting mycelia for amino acids, it was considered important not to add organically bound nitrogen to the substrates. The employed medium was patterned according to the one described by Stoll, *et al.* (5), in their successful work with ergot isolated from *Pennisetum typhoideum* Rich., and consisted of the following:

Mannitol	50.0 Gm.
Sucrose CP	20.0 Gm.
Potassium dihydrogen phosphate	1.0 Gm.
Magnesium sulfate $7H_2O$	0.3 Gm.
Manganese sulfate	0.10 Gm.
Ferrous sulfate $7H_2O$	0.004 Gm.
Succinic Acid	5.4 Gm.
Distilled water to make	1,000 cc.

This medium, which was slightly acidic, was subsequently adjusted to a pH of 5.6, by use of approximately 4 ml. of stronger ammonia solution per 1,000 ml. Thus, NH_3 was the only nitrogen source of the fungus. The produced fungal material was processed as described in an earlier publication (3).

Analytical.—The sclerotia and the saprophytic fungal material were subjected to the same analytical procedure following in principle that of Grøger and Mothes (6): 1.000 Gm. of dried substance was extracted exhaustively with petroleum benzin to remove all lipid material. The marc was subsequently extracted with 20 ml. of 50% ethanol overnight in a horizontal shaking machine. The resulting filtered extract was evaporated to dryness in a Virtis freeze drier. The isolated soluble material was then dissolved in 0.5 ml. of a mixture of ethanol and water (4 + 2 by volume) to which was added one drop of chloroform as a preservative. The resulting solution was subjected to paper partition chromatography, ascending formation being employed. Several solvent systems recommended in the literature (7) as satisfactory for amino acid analyses were tried; but with the majority, relatively poor separation was obtained. The most satisfactory solvent system was found to be in the first direction azeotropic aqueous *n*-propanol (71.7 % w/w). In the second direction, a mixture of 80% phenol in water was employed. The chromatograms were sprayed routinely, after drying, with ninhydrin solution (7), and all identified spots were substantiated by cochromatography with authenticated amino acids.

RESULTS AND DISCUSSION

In the saprophytic cultures polymorphism was less pronounced than in the previously employed media (3). Growth was relatively slow, as evidenced by a lag phase of seven days. The fungal mat was brownish blue in color, and a microscopic inspection revealed an abundant formation of conidia in all five parallel experiments. A test for sucrose in the culture filtrate was negative; the pH had decreased to 5.0, indicating a slight acidification during cultivation. The yield of dried fungal material was more uniform than experienced in previous work (3) and ranged from 0.913 to 1.183 Gm. per 70 ml. of medium.

The lipid production showed a significant difference in the two ergot samples. The sclerotia contained 17.6% of petroleum ether soluble substance as against 12.3% in the saprophytically developed fungal material. This finding may be due to the difference in cultivation temperature. The sclerotia were developed on free land during the hot Texas

* Received November 10, 1958, from the College of Pharmacy, University of Texas, Austin.

summer, while the artificial cultivation took place under constant temperature in an air-conditioned laboratory.

The results of the amino acid study are illustrated in Figs. 1 and 2. Several ubiquitously occurring amino acids were identified, however, no appreciable qualitative difference was found between the artificially and the parasitically developed ergots. Spots 9 and 13 are unidentified and are probably polypeptides.

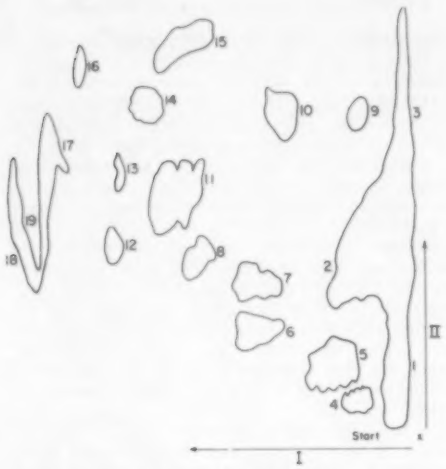

Fig. 1.—Free amino acids in sclerotia.

I, Azeotropic *n*-propanol. *II*, Phenol-water (80 + 20). *1*, Lysine. *2*, Arginine. *3*, Histidine. *4*, Aspartic acid. *5*, Glutamic acid. *6*, Serine. *7*, Glycine. *8*, Threonine. *9*, Unidentified. *10*, α-Aminobutyric acid. *11*, Alanine. *12*, Tyrosine. *13*, Unidentified. *14*, γ-Aminobutyric acid. *15*, Valine. *16*, Proline. *17*, Isoleucine. *18*, Phenylalanine. *19*, Leucine.

Quantitatively, there seemed to be a marked difference with respect to the metabolism of alanine and glutamic acid as judged by the size of the test spots. Hyphae produced the greatest quantity of alanine, while sclerotia on the other hand were richer in glutamic acid.

Spots 1, 2, and 3 were identified by cochromatography with known pure amino acids; however, it is highly possible that these diffuse spots also represent various proteins which react with ninhydrin. A study of the latter is presently under way.

The most striking result of our investigation is that we were unable to ascertain in any culture the presence of tryptophan, which in many publications has been suggested as the precursor of all ergot alkaloids (3). Its detection was attempted with several specific chromatogram developers for tryptophan, as listed in current literature (7). This finding is in agreement with that of Grøger and Mothes (6), and also in line with the result of

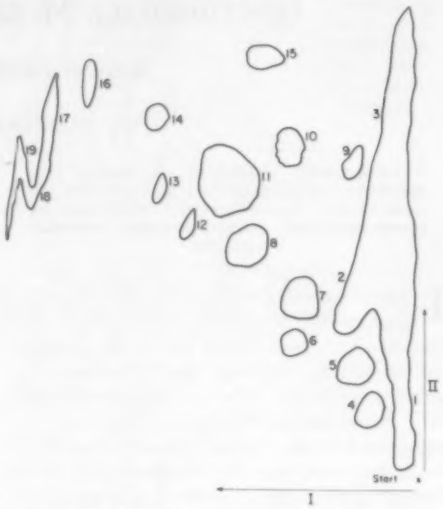

Fig. 2.—Free amino acids in saprophytic fungal material.

I, Azeotropic *n*-propanol. *II*, Phenol-water (80 + 20). *1*, Lysine. *2*, Arginine. *3*, Histidine. *4*, Aspartic acid. *5*, Glutamic acid. *6*, Serine. *7*, Glycine. *8*, Threonine. *9*, Unidentified. *10*, α-Aminobutyric acid. *11*, Alanine. *12*, Tyrosine. *13*, Unidentified. *14*, γ-Aminobutyric acid. *15*, Valine. *16*, Proline. *17*, Isoleucine. *18*, Phenylalanine. *19*, Leucine.

Suhadolnik and Loo, who, according to a recent publication (8) on ergot failed to obtain radioactive alkaloids *in vivo* by use of tagged tryptophan as precursor. Thus, it may seem justified to conclude that tryptophan is not directly involved in the biosynthesis of lysergic acid or the ergot alkaloids. The absence of sulfur containing amino acids is also noteworthy, although no speculations as to its significance will be offered at this time.

The abundant occurrence in both types of fungal material of the aminobutyric acids indicates that these hardly are involved in the alkaloid biosynthesis. Since no organically bound nitrogen was present in the saprophytic media, a strong anaboliti. capacity of *Claviceps* for N is in evidence.

Studies of the comparative metabolism of carbohydrates, organic acids, lipids, and polypeptides of ergot are in progress.

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Ion Exchange Separation Method for Microdetermination of Tropane Alkaloids in the Presence of Morphine*

By EERO SJÖSTRÖM† and ANTTI RANDELL

A method has been developed for the quantitative determination of micro amounts of atropine and scopolamine in the presence of large quantities of morphine. The components are first separated on an anion exchange column and the tropane alkaloids then colorimetrically determined. It is found that when solutions of relatively high methanol concentrations are used the tropane alkaloids are not hydrolyzed by the strongly basic resin. By contrast, in aqueous solution, a considerable saponification takes place and the results obtained are consequently too low. The method has been applied to the determination of tropane alkaloids in injection solutions. The method is simple and gives very satisfactory accuracy and reproducibility.

SEVERAL ANALYTICAL ion exchange methods have been reported during recent years for isolation of morphine and other phenolic alkaloids from different materials (1–7) as well as for separation of morphine from nonphenolic alkaloids (8–12). The latter methods are based on the fact that morphine is quantitatively retained in a column filled with a strongly basic resin, whereas the nonphenolic alkaloids pass through. Since the alkaloid salts are converted into the corresponding free bases, they can be titrated in the effluent. According to this principle codeine and atropine, for instance, can be determined in the presence of morphine.

Although the ion exchange-titration methods can be used in many cases, their applicability is limited to relatively high concentrations. Yet, in medical preparations the tropane alkaloids are often present as a microcomponent. In these cases the titration becomes very inaccurate. Furthermore, the inaccuracy is seriously increased with increasing ratios of morphine/atropine (or scopolamine) for the following reasons: (*a*) "pure" morphine of pharmacopeial quality usually contains up to 0.5% of nonphenolic alkaloids which are titrated with the tropane alkaloids, (*b*) ionized substances such as the neutral salts, are also exchanged and titrated with the tropane alkaloids, and (*c*) it is difficult to avoid traces of alkaline impurities dissolved from the resin even when working carefully under controlled conditions.

The object of the present investigation was to develop a method for quantitative microdetermination of tropane alkaloids in the presence of large amounts of morphine. For the reasons given above, it was evident that the determination after the separation must be based on some more sensitive and specific procedure than titration. The methods commonly used for this purpose are colorimetric and are based on nitration of the tropic acid portion of the tropane alkaloid molecule. Accordingly, in using these methods it is essential that no saponification should take place in the resin, otherwise the tropic acid liberated is retained in the column. Experiments showed that when the alkaloids are passed through the column in aqueous solution a considerable saponification occurs. On the other hand, it was found that if methanol or ethanol solutions are used, no saponification takes place and the alkaloids pass quantitatively into the effluent. Since morphine is completely retained by the resin even from these solutions, it was possible to devise a method suitable for the microdetermination of tropane alkaloids in the presence of morphine.

EXPERIMENTAL

Equipment and Materials.—The experiments were performed with the commercial resin Dowex 1 with various degree of cross-linking, having round particles of 50 to 100 mesh. The ion exchange columns were of standard type, measuring 6 x 110 mm. The resin was converted into the hydroxyl ion form with about 50 ml. of 1 *N* sodium hydroxide and washed with water to neutral against methyl red. Before the separation experiments the water was displaced from the columns with the solvent (about 50 ml.) and gas bubbles, if they appeared, were removed (17).

The morphine used was of pharmacopeial quality (Ph. F. VII) and purified in the following way. About 300 mg. of morphine hydrochloride was dissolved in 100 ml. methanol and passed through the ion exchange column. The column was afterwards washed with absolute methanol until the nonphenolic impurities had completely passed into the effluent (about 50 ml.). The completeness of the washing was checked by means of ultraviolet measurement, i. e., the column was washed until no absorption could be observed in the ultraviolet. The

* Received December 23, 1958 from the Analytical Laboratory, Lääketehdas Orion Oy, Pharmaceutical Manufacturers, Helsinki, Finland.

† Present address: Stora Kopparbergs Bergslags AB, Central Laboratory, Falun, Sweden.

Grateful thanks are given to Miss Margit Gustafsson for the paper chromatographic analyses and to Mrs. Kirsti Salmela for carrying out the experiments with injections.

morphine taken up was eluted with 0.33 *M* boric acid solution in absolute methanol. After evaporation of the boric acid and the methanol from the eluate, the remaining free morphine was dissolved in ethanol and neutralized with hydrochloric acid. The resulting morphine hydrochloride was recrystallized from ethanol and dried in a vacuum desiccator in the presence of sulfuric acid.

Atropine sulfate and scopolamine hydrobromide of pharmacopeial quality (Ph. F. VII) were used as such. The methanol and all the reagents were from Merck (Darmstadt). The ethanol from Oy Alkoholiliike Ab for spectrophotometric purposes was used.

The measurements were carried out with a Zeiss spectrophotometer, model PMQ II, 1-cm. cells being used.

The Analytical Procedure.—After numerous experiments a suitable procedure for the estimation of atropine and scopolamine was devised by modifying the method described by Bratton and Marshall (13, 14). This method is based on the reduction of the nitrated alkaloid and its subsequent diazotization. The color produced is stable. The resulting procedure is carried out as follows: Transfer an aliquot of the ion exchange effluent containing about 0.06 to 0.14 mg. alkaloid into a 50-ml. beaker and evaporate to dryness on a water bath. Allow to cool and add fuming nitric acid (0.6 ml.) and evaporate the excess of nitric acid on a water bath. Dissolve the residue in 50% ethanol (10 ml.). Add 10% hydrochloric acid (5 ml.) and 0.3 Gm. zinc dust. Cover the beaker with a watch glass and allow to stand for ten minutes on a water bath. Cool the solution to room temperature and filter it into a 25-ml. volumetric flask by washing the residue of zinc with water (2 × 3 ml.). Add 1% sodium nitrite solution (1 ml.), mix, and let stand for ten minutes. Add 2.5% ammonium sulfamate solution (1 ml.), shake, and let stand for ten minutes. Finally, add 1% N-(1-naphthyl)-ethylenediamine dihydrochloride solution (1 ml.), made up to volume with water, and measure after about thirty minutes at 550 mμ. Prepare a blank with the reagents and standards, simultaneously with the sample. At the concentration range mentioned the corresponding absorbance is about 0.3 to 0.7. Since the reproducibility of the results seems to be good, the alkaloid content can even be calculated from a standard curve.

In connection with the saponification experiments atropine was also determined in the ultraviolet region, its maximum being at 211 mμ. For the detection of morphine, ultraviolet measurements were performed at its maximum of 286 mμ.

RESULTS AND DISCUSSION

Uptake of Morphine Hydrochloride.—The break-through curve of the purified morphine hydrochloride in 80% methanol is shown in Fig. 1. As can be seen, the break-through at a flow-rate of 1 ml./min. is relatively sharp even when a normally cross-linked resin Dowex 1X–8 is used. Morphine could not be detected by ultraviolet measurement in the effluent of either of the resins when 70 ml. of 0.5% morphine hydrochloride solution was passed through. Accordingly, within the limits of the measurement sensitivity, not more than 0.02% has passed through the column. Both resins were able

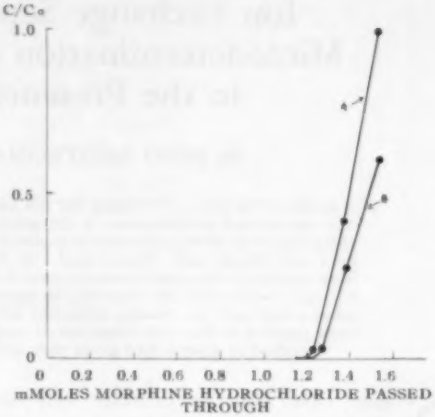

Fig. 1.—Break-through curve for morphine hydrochloride (0.0155 mole/l in 80% (w/w) methanol). Curve 4: Dowex 1X–4. Curve 8: Dowex 1X–8. Total capacities of the columns 3.45 and 3.36 meq.

to take up about 1.2 m*M* (375 mg.) of anhydrous morphine hydrochloride quantitatively, i. e., about 70% of their theoretical total capacity for morphine hydrochloride.

It must be pointed out that when morphine hydrochloride of pharmacopeial quality (Ph. F. VII) was passed through the column, the effluent showed a strong absorption in the ultraviolet. The impurities were identified by paper chromatography. The main impurity, about 0.4% of the original morphine preparation, was proved to be codeine.

Hydrolysis of Tropane Alkaloids by the Resin.—The influence of solvent composition on the saponification of the alkaloids was studied by using methanol and ethanol solutions with various amounts of water.

The experiments were performed by passing 20 ml. (2 mg.) of the alkaloid salt solution through the column, which was afterwards washed with solvent to obtain an effluent volume of 50 ml. The results are summarized in Table I. As can be seen from these data, hydrolysis is quite naturally favored by

TABLE I.—HYDROLYSIS OF ATROPINE AND SCOPOLAMINE IN METHANOL-WATER AND ETHANOL-WATER MIXTURES[a]

Alcohol Concentration, (by Weight) %	Found in Effluent, %: Atropine[b] in Methanol-Water	Atropine[b] in Ethanol-Water	Scopolamine[c] in Methanol-Water
100	100.0	...	97.0
90	98.1	102.0	95.5
80	100.0	...	93.5
70	98.1	96.5	93.5
60	97.8	...	86.5
50	96.5	92.2	79.5
40	89.7	...	66.5
30	79.7	69.5	59.5
20	60.4	...	50.0
10	53.5	21.8	...
0	33.0	...	...

[a] Resin Dowex 1X–4, Flow-Rate 1 ml./min.
[b] Ultraviolet determination. [c] Colorimetric determination.

the increasing water content. Within the limits of experimental error all the atropine passes through the column if the methanol content is 70% or more. On the other hand, in aqueous solution, 67% of the atropine is saponified. It can be noted, too, that scopolamine is more easily saponified than atropine. However, it must be pointed out that the results are not strictly comparable, since the flow-rate could not be kept absolutely constant. Nevertheless, it can be inferred from the experimental data, that for the quantitative recovery of scopolamine the methanol concentration must be kept near 100%.

These results are in good agreement with earlier observations concerning the ester interchange of estradiol benzoate by strongly basic resin. It was namely found that in absolute methanol and ethanol only transesterification takes place and no hydrolysis (15). The reason is that the hydroxyl ions are completely displaced by the alkoxide ions according to the following reaction:

$$MeOH + HO^- \rightleftarrows MeO^- + H_2O$$

The apparent equilibrium constant for this reaction was found to be 11.1 (16). This means that if the methanol concentration in the resin phase is 70%, 97% of the hydroxyl ions of the resin are converted into methoxide ions. The colorimetric method used did not reveal whether the tropane alkaloids are interchanged in the column, since the methyl tropinate possibly formed is coestimated. A paper chromatogram of the effluent indicated, however, that within the limits of the accuracy, i. e., about ±10%, no ester interchange takes place.

Separation of Tropane Alkaloids From Morphine.—In order to check the effectiveness of the separation method, alkaloid mixtures varying in the ratio of morphine/atropine (or scopolamine) were passed through the column. The experiments were performed by passing 20 ml. of the alkaloid salt mixture through the column, which was afterwards washed with solvent to obtain an effluent volume of 50 ml. The results obtained (Tables II and III) may be considered satisfactory even though the deviations become greater when the tropane alkaloid content is extremely low compared with the morphine present. The errors probably arise from the impurities dissolved from the resin and from the nonphenolic impurities of the morphine.

Elution of Morphine.—The column can be regenerated with a 0.33 *M* boric acid solution in absolute methanol. This solution was earlier observed to be an excellent eluting agent for estradiol (17, 18). The results obtained in the present investigation show that boric acid in methanol is also very effective for displacing morphine. This observation is of importance as difficulty has earlier been reported in this process (19, 20). Apart from its elution efficiency, the methanolic boric acid has the added advantage that it evaporates readily. The elution curves for different resins with various degree of cross-linkage are illustrated in Fig. 2. It can be noted that morphine is, quite naturally, more easily displaced from the resins with a low degree of cross-linkage. However, a good recovery is also achieved by using normally cross-linked resins. It is interesting to note that by substituting methanol for ethanol, the elution efficiency is markedly decreased, as can be seen from Figs. 3 and 4.

Application of the Method to Medical Preparations.—The method has been used with success in this laboratory for a period of half a year. At the beginning, the Vitali reaction was used for the determination of tropane alkaloids. However, when it was found that the reproducibility was often poor, the method was changed to that described in this paper. The ion exchange separation combined with this method has proved to be of very satisfactory accuracy and reproducibility. Some typical data are collected in table IV.

It may be emphasized that glycerol, which is often

TABLE II.—SEPARATION OF ATROPINE FROM MORPHINE[a]

Added		Found in Effluent, Atropine Calculated as Sulfate,	
Morphine Hydrochloride, mg.	Atropine Sulfate, μg.	μg.	Error, %
50	1,000	945	−5.5
50	500	500	±0.0
50	200	197	−1.5
100	200	204	+2.0
100	100	116	+16.0

[a] Dowex 1X–4, 80% (w/w) methanol, Flow-Rate 1 ml./min.

TABLE III.—SEPARATION OF SCOPOLAMINE FROM MORPHINE[a]

Added		Found in Effluent, Scopolamine Calculated as Hydrobromide,	
Morphine Hydrochloride, mg.	Scopolamine Hydrobromide, μg.	μg.	Error, %
50	1,000	975	−2.5
50	500	510	+2.0
50	200	209	+4.5
100	200	216	+8.0
100	100	106	+6.0

[a] Dowex 1X–4, Absolute Methanol, Flow-Rate 1 ml./min.

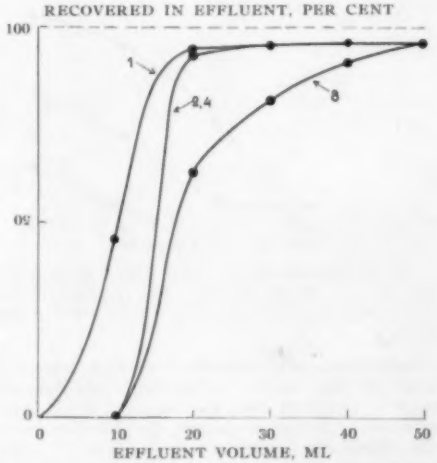

Fig. 2.—Elution of morphine with 0.33 *M* boric acid in absolute methanol. The numbers of the curves indicate the cross-linking of the corresponding resin.

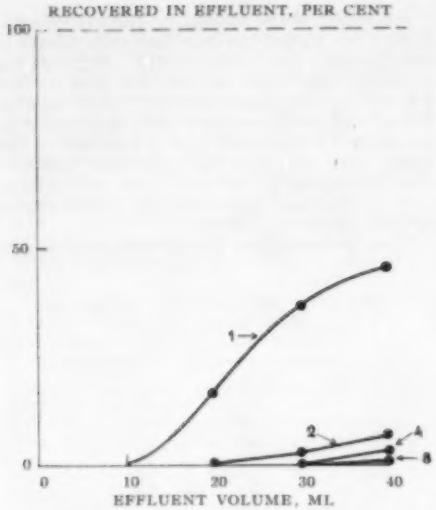

Fig. 3.—Elution of morphine with 0.33 *M* boric acid in absolute ethanol. The numbers of the curves indicate the cross-linking of the corresponding resin.

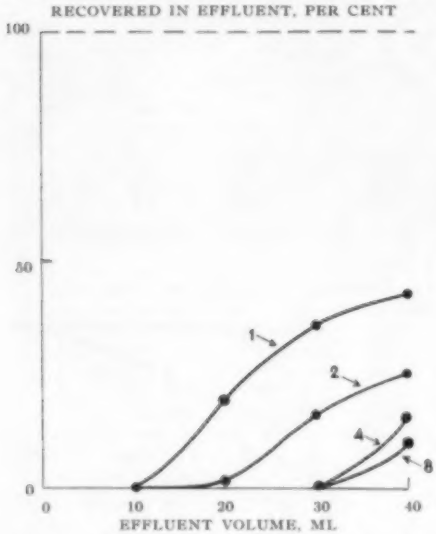

Fig. 4.—Elution of morphine with 0.33 *M* boric acid in 70% (w/w) ethanol. The numbers of the curves indicate the cross-linking of the corresponding resin.

present in injection solutions of this type, interferes with the colorimetric estimation. Therefore, it must be separated from the mixture. If an anion exchange column alone is used, glycerol passes into the effluent along with the tropane alkaloids. This problem has been solved by coupling a cation exchange column in series with the anion exchange column. Thus, the morphine is first removed by the anion exchanger, the tropane alkaloids passing with

TABLE IV.—DETERMINATION OF ATROPINE AND SCOPOLAMINE IN INJECTIONS

Composition and Content, mg.		Atropine or Scopolamine Found Calculated as Respective Salts mg.	Error, %
Morphine hydrochloride	20	0.250	±0.0
Atropine sulfate	0.25	0.275	+10.0
Glycerol	50	0.250	±0.0
Alcohol	120	0.260	+4.0
Distilled water ad.	1 ml.		
Morphine hydrochloride	20	0.580	−3.3
Scopolamine hydrobromide	0.6	0.561	−6.5
Glycerol	50	0.580	−3.3
Alcohol	120	0.580	−3.3
Distilled water ad.	1 ml.		

the glycerol into the cation exchange column. Glycerol can easily be washed from the column with the solvent. The tropane alkaloids taken up by the cation exchanger are finally eluted with a methanol solution containing ammonia and determined in the eluate. The details of the resulting procedure are as follows: Pipet a suitable amount of the injection solution (2–4 ml.) into a beaker and add absolute methanol (100–150 ml.). Transfer this solution through an anion exchanger column and let the effluent pass directly into a cation exchange column of the same size (Dowex 50X–4, H^+-form, 50/100 mesh). Wash the anion exchange column with absolute methanol (about 50 ml.) and also let the washing methanol pass through the cation exchanger. Elute the tropane alkaloids from the cation exchanger with a solution which is prepared by diluting 60 ml. strong ammonia solution to 100 ml. with methanol, and let the eluate pass directly into a 100-ml. volumetric flask. Take from this solution an aliquot (10 ml.), evaporate the solvent on a water bath, and determine the alkaloid content as described.

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An Examination of the Oil of the Seeds of Persimmon (*Diospyros Virginiana* L., Fam. *Ebenaceae*)*

By CECIL P. CLOUGHLY and HENRY M. BURLAGE

A fixed oil was extracted by petroleum ether from the seeds of *Diospyros Virginiana* L., representing approximately three per cent of the weight of the seeds. The oil is semidrying, and its properties are similar to sesame oil. The petroleum ether extract has a dark, reddish brown color, musk-like odor, and a bland taste like olive oil. It consists of 80.24 per cent unsaturated fatty acids, of nearly equal parts of oleic and linoleic acids. The saturated fatty acids consist of stearic and palmitic acids, with traces of myristic and arachidic acids. Saturated acids amounted to 16.25 per cent of the total weight of the oil. The amount of unsaponifiable matter and the physical constants were determined. A qualitative identification of the saturated fatty acids was made by chromatography using the technique developed by Holasek and Winsauer. Separation of the saturated and unsaturated fatty acids was effected by Pelikan and Von Mikusch modification of the Bertram oxidation method. Stearic and palmitic acids were identified after their separation by fractional crystallization from methanol at low temperatures. Results indicate that: The chemical constituents of the oil of the seeds of *Diospyros Virginiana* L. are similar to sesame oil; yield of the oil from the seeds is 3.41 per cent; and possibility exists that this oil might be useful in the formulation of perfumes or cosmetics where the oil might be used to advantage.

ALTHOUGH the persimmon tree is a familiar part of the countryside in the South, it has never been extensively cultivated in this country. It is considered by some persons to be an undesirable plant, and studies have been made to destroy the tree by poisons when it is discovered growing voluntarily (1).

DeSoto, in 1539, learned from the Indians the value of persimmon fruit as a supplement to his diet, and he remembered this in the narrative of his expedition written eighteen years later at Evora in 1557 (2).

The common name, persimmon, is of Virginia Indian origin, and its longer, botanical name is *Diospyros Virginiana* L. There are approximately 160 varieties of *Diospyros*, mostly Asiatic. The common American species ranges southward from southern Pennsylvania to the Gulf Coast and westward to Texas. It is also found in southern New York, West Virginia, Ohio, Indiana, Illinois, southern Iowa, Missouri, and Kansas. In Florida the tree is sometimes called "possum wood" because the fruit attracts the small mammal.

The tree produces what is technically a berry, and is described as being subglobose, varying to depressed or elongate, 3 to 4 cm. in diameter. The fruit has one to eight seeds that are flat, elliptic, and very hard. The main characteristic of the fruit is that it is exceedingly astringent when green, but sweet and edible when ripe or after exposure to frost.

The leaves of the tree look something like the American magnolia tree, and are four to six inches long, thick, and oval. The leaves in summer are a shiny dark green, but during the autumn they become brilliant orange or scarlet, adding much to the beauty of the countryside.

The bark of the tree is nearly black and is divided into squares. The tree grows to an average height of 50 feet, although there are some exceptions as noted in Illinois and Indiana of persimmon trees growing as tall as 100 feet. It spreads from roots and often covers abandoned fields and roads with a scrubby growth. It also grows well from seeds and is propagated by migratory birds. The wood is very hard and dense, and after the tree has matured a hundred years, the wood is known as "heartwood." This is used in making valuable hardwood objects. As previously noted, the fruit of this tree has been cultivated in Asia since ancient times, and many varieties of persimmons have been developed (3).

EXPERIMENTAL

Preliminary Treatment of Seeds.—Seeds for the study were taken from the forests of trees of *Diospyros Virginiana* L., growing in Orange County, North Carolina. The meaty material of the fruit that clung tenaciously to the seeds was removed by rubbing vigorously between hard-finished pieces of cloth. The seeds were reduced to a moderately coarse powder in a Wiley grinding mill and then treated with a series of selective solvents in succession in a continuous extraction apparatus in the following order: petroleum ether, absolute ether, chloroform, U. S. P., absolute alcohol, and 70% ethyl alcohol.

The research was confined to the petroleum ether extract since examination of the other extracts showed that they contained materials removed

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from the seed body but no oil. After the petroleum ether was removed under controlled conditions, a preliminary examination of the resultant substance showed that the dark reddish-brown oil (3.41%) was soluble in the usual organic solvents and was readily saponified. Elemental analysis showed negative tests for nitrogen, sulfur, halogens, and phosphorus.

Persimmon seed oil is classified with the semidrying oils (4). The Hehner and Mitchell hexabromide test (5) gave negative tests for linolenic acid. Figure 1 shows the percentage increase in weight of persimmon seed oil and laboratory samples of linseed and sesame oil when exposed to the atmosphere under controlled conditions. Chemical properties of the oil obtained by following standard methods (6, 7, 8) are shown in Table I.

The infrared spectrum of persimmon seed oil showed no unusual infrared absorption bands produced by the oil at particular wavelengths.

Partition Chromatography of Saturated Fatty Acids from Persimmon Seed Oil.—The soap solution remaining after saponification of a weighed sample of the oil was dissolved in hot distilled water and treated with diluted sulfuric acid until neutral to phenol red. The water-insoluble fatty acids were separated from the mixture by extraction with petroleum ether. An aliquot portion of this extract was measured, the solvent evaporated off, and the residue of fatty acids then accurately weighed. The concentration of the fatty acids in the petroleum ether extract was next adjusted so that 0.35 Gm. of such acids were present in each 25 ml. of solution, and a method was sought to identify the acids by use of paper chromatography. This concentration was chosen because it would furnish approximately 66 μg. of acids in each drop of solution to be used in the procedure.

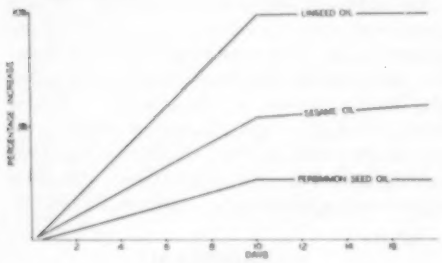

Figure 1.

TABLE I.—PROPERTIES OF PERSIMMON SEED OIL

Elemental Analysis	
Nitrogen	Absent
Sulfur	Absent
Halogens	Absent
Phosphorus	Absent
Refractive Index (25°)	1.4800–1.4801
Acid value	30.3
Ester value	129.3
Iodine value	104.01
Saponification value	160.23
Thiocyanogen number	78.04
Unsaponifiable matter	4.9%
Oxygen absorption—Gain in weight of 2.8/100 parts in ten days.	

The solution of fatty acids was transferred by a lambda pipet to strips of paper impregnated with 0.5% $KAl(SO_4)_2.12H_2O$. These strips were then developed in standard chromatography jars according to the method of Holasek and Winsauer (9). The developer used consisted of approximately 300 ml. of a solution of freshly distilled carbon tetrachloride, freshly distilled methyl alcohol, and concentrated ammonia (81:18:1). After developing for twelve to fourteen hours, the strips were removed, dried in an oven at 60° for twenty minutes, and then sprayed with a 0.1% solution of Rhodamin B in 0.05 *N* hydrochloric acid. The papers were again dried in the oven and examined under ultraviolet light. Separated fatty acids showed up as dark red spots in a red to violet background. Persimmon seed oil fatty acids developed spots on such chromatograms with R_f's of 70, 80, and indefinite spots at R_f's of 65 and 90. This indicated that palmitic and stearic acids were present, and possibly myristic and arachidic acids.

Separation of Saturated from Unsaturated Fatty Acids.—Separation of the saturated from the unsaturated fatty acids was accomplished by the Pelikan and Von Mikusch modification of the Bertram oxidation method (10). Using this method, a sample of persimmon seed oil weighing 5.281 Gm. yielded 0.9048 Gm. of saturated fatty acids, and represented 17.1% of the total weight of the oil. Since the isolated fatty acids often carry over small portions of unsaturated fatty acid, it was necessary to perform a correction to the first calculation of saturated fatty acids. The iodine value of the isolated fatty acids was computed by the U. S. P. method (11) and was found to be 4.6.

The following formula was used to calculate the corrected value for the saturated fatty acid content of the oil:

$$G = 100/S \quad W = IW/90$$

where G = corrected saturated fatty acids content in %, W = weight of the isolated fatty acids (0.9048 Gm.), I = iodine value of the isolated fatty acids (4.6), and S = weight of the sample used (0.9048).

By substitution in the above equation, it was determined that the correct percentage of saturated fatty acids in persimmon seed oil amounted to 16.26 % (10). See Table II.

TABLE II.—FATTY ACID COMPOSITION OF PERSIMMON SEED OIL

Saturated fatty acids present: arachidic (trace), myristic (trace), stearic and palmitic	16.26%
Unsaturated fatty acids:	
Linoleic acid (calculated as glycerides)	39.36%
Oleic acid (calculated as glycerides)	40.88%

Determination of Unsaturated Fatty Acids.—The thiocyanogen and iodine values, both previously determined, were used to calculate the percentages of hypothetically pure glycerides in persimmon seed oil (12). By using these values and a formula recommended by the Association of Official Agriculture Chemists (13) it was determined that persimmon seed oil consisted of the following unsaturated fatty acids: linoleic acid, 39.36% and oleic acid, 40.88%.

Separation of the Saturated Fatty Acids.—The saturated fatty acids remaining from the permanganate oxidation experiment were separated from each other quantitatively by fractional crystallization from methanol at low temperatures (14). Palmitic and stearic acids were identified in the fractions by mixed melting points.

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Air-Suspension Technique of Coating Drug Particles*

A Preliminary Report

By DALE E. WURSTER

A new method of rapidly coating drug particles of widely varying size and shape is presented. In this process, the drug particles are coated and dried while suspended in an upwardly moving current of air. Solutions and suspensions of coating materials in both water and volatile organic solvents are employed. The drying of the coated particles is accomplished at either room or elevated temperatures, depending on the solvent used. Some fundamentals and an introduction to practical applications of the process are given.

CONSIDERABLE EFFORT has been expended to develop more rapid and more precise methods of coating compressed tablets and other drug particles. Progress in this field includes refinements of the conventional pan coating method and compression coating. The process described in this paper is a departure from both of the previous methods. It is simple, extremely rapid, and appears to be adaptable for large volume coating operations.

APPARATUS

The apparatus consists of a vertical column which is constricted at the bottom and expanded at the top (Fig. 1). The air velocity in the constricted portion of the column is so great that particles entering this region are immediately propelled upwards. In the expanded portion of the column the air velocity is greatly decreased. This decreased velocity will not support the particles and they fall to the central or working region of the column. In Fig. 1 it can be observed that the column also contains a side vent. Both the side vent and constricted portion of the column contain adjustable plates with which the velocity of the air stream in the working region of the column can be controlled. Various modifications in the design of the column can be employed; however, the upper portion is usually expanded to decrease the

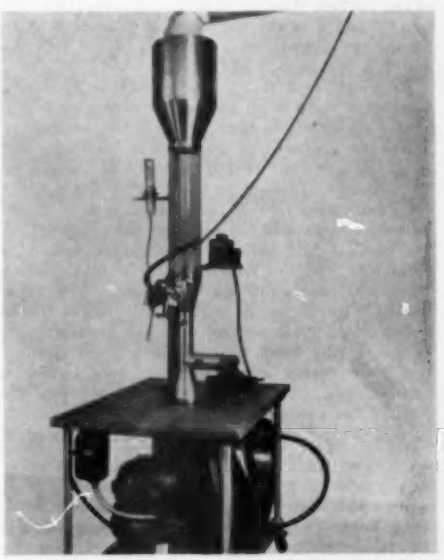

Fig. 1.—Air-suspension coating apparatus.

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velocity and prevent the loss of particles. Various segments of the column may also be selected as the coating region but the velocity must be controlled to confine the particles to this region. A constant speed blower (Buffalo Forge Co. Type 5E) positioned at the base of the column serves as the air source. A thermostatically controlled gas burner is utilized to heat the air prior to its entrance to the blower. An atomizer for spraying the coating solution is normally positioned immediately below the working region of the column, but its position can be varied for specific uses.

PROCESS

The process consists simply of supporting particles in a vertical column with an upwardly moving air stream during which time the coating solution is atomized onto the suspended particles. The velocity of the air stream is adjusted so that the suspended particles are maintained in a relatively confined working region of the column. The drying time of the coat can be governed by controlling either the atomization rate, the temperature of the supporting air stream, or both.

A simple method for approximating the velocity required to suspend spherical particles in air was found to be useful in this work. Since the particles are suspended in an air stream rather than just agitated on a supporting surface but still are not transported, the particles were compared on the basis of their weight and cross-sectional area to equivalent inches of water (static pressure). The velocity required to support the particle was therefore calculated as follows:

$$V = K\sqrt{M/(A/k)}$$

where V is the velocity in feet per minute, K the velocity required to maintain a static pressure of one inch of water (4,005 ft./min.). M is the weight of the particle (Gm.). A is the cross-sectional area ($cm.^2$), and k is the weight of a column of water having the dimensions 1 cm. x 1 cm. at the base and 2.54 cm. high (2.54 Gm.).

It is apparent that particles can be held in a suspended state in an upwardly moving current of air for an indefinite time period if the velocity required to support the particles is held constant. It is also apparent that if particles in the column are uniformly exposed to the coating solution and the atomization rate of the solution held constant, a linear relationship between the gain in weight of the particle and time of atomization should be obtained. This, of course, assumes that possible attritional effects are not significant. The thickness of the coat on the particle will not, of course, increase in a linear manner since the specific weight of the coating solids obtained in a given atomization time period must cover an ever increasing surface area. A linear relationship between the cube of the radius of the particle or the increase in volume and time should, however, be obtained.

EXPERIMENTAL

Air Velocity. Since the vertical column contains adjustable plates to control the air stream, the column was first calibrated in order to determine the average velocity obtained in the column for each setting of the plates. The average column velocities were determined with a pitot tube and an Ellison (2 inch) inclined draft gauge.

Large hollow spheres were employed to study the average velocity required to suspend spherical particles. The density of the particles was increased by injecting a given quantity of water into them. The particles were then accurately weighed. In this manner the density of the particles was increased but the effective area upon which the upward moving air impinged was held constant. The average velocity required to suspend the sphere was then obtained from the column calibration curve.

Studies on Coat Build-Up.—Small, uniform, spherical sugar particles were initially coated with "Carbowax" 6000 in order to increase the particle size sufficiently so that they could be easily measured with a micrometer caliper. The average particle size was determined and a known weight of these particles was placed in the coating column. The particles were coated with a solution containing 25% "Carbowax" 6000 (w/v) in methyl alcohol. The atomization rate was maintained at approximately 56.34 ml. per minute (2,000 ml. atomized in thirty-five and one-half minutes). Due to the volatility of the solvent the column was operated at room temperature. At the end of the above time interval the contents of the column were removed, spread out, and allowed to dry at room temperature (approx. twenty-four hours) until a constant weight for the batch was obtained.

The average weight of the coated particles obtained in each test was determined from five samples, each containing 150 particles.

The average particle diameter was obtained by measuring the individual particles in the above 150 particle samples with a micrometer caliper.

Coating Procedure.—The velocity required to suspend compressed tablets and the smaller particles was first calculated in order to obtain the approximate column setting and was then determined experimentally. Two to four kilos of the particles to be coated were introduced into the column and atomization of the coating solution started as soon as the particles reached the operating height in the column. In the case of compressed tablets the lower half of the Lucite tube (see Fig. 1) was the working height, whereas with smaller particles, the entire length of the tube was employed. The particles were immediately removed from the column after the desired volume of coating solution had been applied.

When aqueous solutions were employed the column was operated at a temperature of 49–50° and the coating solution was maintained at 85–90°. With volatile solvents the column was operated at room temperature.

Solutions employed for applying rounding coats to compressed tablets included: simple syrup; simple syrup, 67% and 33% of a "Carbowax" 4000 solution (50% w/v); simple syrup, 50% and 50% of a "Carbowax" 4000 solution (50% w/v); and 56.5% simple syrup, 19.8% starch paste (4.25% w/v), 22.6% "Carbowax" 4000 (50% w/v), and 1.1% Tween 20. These syrups were atomized at a rate of 47 to 48 ml. per minute. Two liters were thus applied in approximately forty-two minutes.

Suspensions of insoluble powders in the syrups were also successfully applied.

Solutions containing 25% wax in carbon tetrachloride were atomized at rates of 50 to 90 ml. per minute on 16–20 and 30–35 mesh particles. Solutions containing 20 or 30% of a lipoprotein or a resin in isopropanol were atomized at rates of 10 to 20 ml. per minute on 40–80 mesh particles.

DISCUSSION AND DATA

Air Velocities.—The experimental velocities required to suspend large spheres of varying density were compared with the velocities calculated according to the given equation. According to the equation a linear relationship should exist between the square root of M/A and the velocity. In this respect a plot of the experimental findings is in fair agreement with a plot of the calculated values (Fig. 2). It is emphasized here, however, that regions of both high and low velocities exist in this particular column. The experimental values given here are thus average velocities.

A single spherical particle in the column was maintained at a uniform height; however, during the actual coating process the suspended particles rise in the high and fall in the low velocity regions of the column. Nevertheless, the velocity requirements for the process were approximated according to the described method.

Coat Build-Up.—In Fig. 3 the average weight of the particle is plotted against the time and indicates that a straight line relationship between the weight of the particle and the time of atomization can be obtained with the process. In Fig. 4 the cube of the radius is plotted against time and again shows a straight line relationship. This tends to indicate that the adherence of the coating solids and the coat build-up occur in a uniform manner.

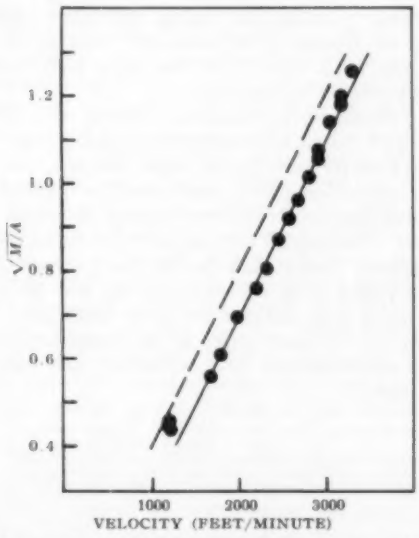

Fig. 2.—Supporting velocity for spheres of varying density. (– – – –Calculated.——Experimental.)

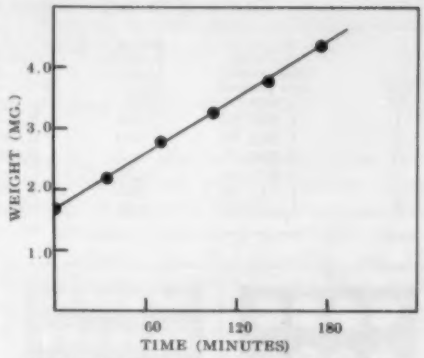

Fig. 3.—Average weight of particles *vs.* time.

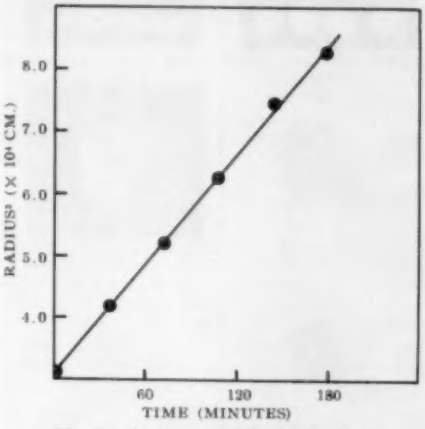

Fig. 4.—Average particle size *vs.* time.

Loss of Solids.—Only a relatively small amount of the atomized solids was lost in the operation of the column when the small sugar particles were coated. This, no doubt, is due to the fact that the extremely large number of particles in the column makes the escape pathway of the atomized particle a very tortuous one. Thus, it is almost impossible for the atomized particle to traverse the length of the column without impinging upon the surface of the particles to be coated. Only a small amount of the atomized material collected on the wall of the column. In Table I it can be observed that less than 1% of the starting material is lost when the loss is calculated on the basis of the total solids introduced into the column. When the loss is based solely on the atomized solids the loss is considerably larger (approx. 3–7%). This latter value, however, appears to be erroneous since the column was not originally designed for small particles and some of particles as well as the atomized solids were lost in the column joints. This is particularly obvious in the last three tests in the table where only one-half the weight of atomized solids was introduced into the column and the per cent loss is approximately two times that of the previous tests. This tends to indicate that the amount of particles lost in the column joints was

TABLE I.—LOSS OF MATERIAL IN COLUMN

Test No.	Weight of Particles Introduced into Column, Gm.	Weight of Solids in Atomized Solution, Gm.	Total Solids Introduced into Column, Gm.	Total Solids Recovered from Column, Gm.	Loss of Total Solids, %	Loss if Based on Atomized Solids, %
1	1,500.00	500	2,000.00	1,983.02	0.85	3.39
2	1,982.66	500	2,482.66	2,470.10	0.51	2.51
3	2,469.68	500	2,969.68	2,950.40	0.66	3.85
4	1,475.20	250	1,725.20	1,709.00	0.94	6.48
5	1,708.42	250	1,958.42	1,938.90	1.00	7.81
6	1,938.24	250	2,188.24	2,172.20	0.74	6.41

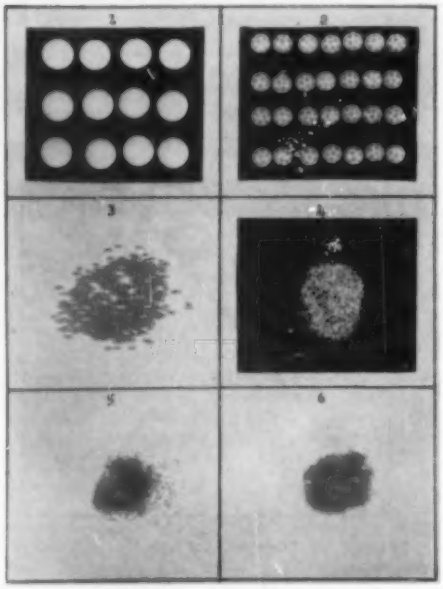

Fig. 5.—Coated particles of various sizes. 1, rounding coat on compressed tablet; 2, compressed tablet made from granulation prepared by coating procedure; 3, wax-coated particles (16–20 mesh); 4, wax-coated particles (35–40 mesh); 5, lipoprotein-coated particles (40–80 mesh); 6, resin-coated particles (40–80 mesh).

the same as in the previous tests and distorts the true value of the atomized solids lost.

Practical Applications.—This paper includes only exploratory work in the realm of practical applications. Subsequent papers will deal more specifically with this phase of the work. However, the process seems to be well suited for applying rounding coats to compressed tablets and for coating smaller particles with a variety of materials.

The rounding coats on tablets were applied in a remarkably short time (twenty-five to forty-five minutes). Simple syrup yielded a highly crystalline and somewhat rough coat: however syrups which formed a more amorphous coat gave a smoother product. It is believed that refinements of the atomization system and formulation changes will make it possible to apply the finishing coats to tablets by this method.

Since a wide variety of substances can be applied by this method small particles can be coated for many purposes such as to change the rate of drug release from the primary particle, to inhibit the chemical decomposition of drugs, and to modify flow properties. Other exploratory work indicates that film type coats on compressed tablets and tablet granulations can be prepared by this method. Also, materials such as those used in compression coating can be applied to drug granules and tablets prepared with a single compression operation with an ordinary tablet machine.

Figure 5 shows particles of several sizes which were prepared by this process.

SUMMARY

A new air-suspension method for the rapid application of coating materials to small particles and compressed tablets is described.

The described process does not appear to be limited by either the coating materials or the solvent system, as both polar and nonpolar materials and solvents have been successfully employed in the coating fluid.

Preliminary investigations indicate the described process to be suitable for applying rounding coats for sugar-coated tablets, film type coats to compressed tablets, and protective coats on small particles to prevent chemical decomposition. The process also appears to be useful for applying coating materials for the purposes of controlling drug release, improving flow properties of small particles, preparing tablet granulations, and many other coating operations in the pharmaceutical, food, animal feed, and other industries.

Synthesis and Antifungal Studies on Sorbic Acid Derivatives*

By DALE H. CRONK†, LOUIS C. ZOPF, and JAMES W. JONES

Nine esters, one amide, and two amine salts of sorbic acid were prepared and tested against four pathogenic fungi by two methods. Of the twelve compounds, *o*-hydroxyphenyl sorbate, chlorothymol sorbate, isobutylamine sorbate, and morpholine sorbate exhibited the greatest activity.

MANY COMPOUNDS have been prepared and tested for antifungal activity. At the onset, the greatest interest was centered on compounds which would protect agricultural products against fungi which attacked the living plants or the crops derived therefrom to produce diseased plants or spoilage in the crop. In more recent years, attention has been given to compounds for the control of fungal-incited disorders in man and other animals. Among the compounds investigated are the fatty acids and their salts or organic derivatives. Rigler and Greathouse (1) studied the antifungal properties of fatty acids in connection with the resistance of plants to certain fungal diseases. They found that antifungal activity increased as the length of the carbon chain increased up to C_{11}, after which activity decreased. Geigy (2) reported that certain amides of α,β-unsaturated acids possess antifungal activity. Further studies on unsaturated acids led to the use of salts of undecylenic acid in the treatment of athletes foot and other dermatoses caused by fungi.

Sorbic acid, which is intermediate in the fatty acid series in respect to carbon chain length and is unsaturated at the α,β and γ,δ-positions, has been approved for the prevention of certain mold growths in foods, beverages, and cosmetic products. Since little work has been reported on the use of sorbic acid or its derivatives in the control of fungi which produce dermatoses or internal disorders in humans, the purpose of this investigation was to prepare certain derivatives and submit them to a preliminary screening against four strains of fungi which are known to be pathogenic. The fungi used were: (*a*) *Microsporum canis*, which is responsible for approximately 10 per cent of the cases of ringworm of the scalp (tinea capitis) among children in the United States. It also attacks glabrous skin. (*b*) *Microsporum gypseum*, which can produce tinea capitis and/or ringworm of the glabrous skin, but is not a common causative agent. (*c*) *Microsporum audouini*, the chief causative agent of tinea capitis among children. It also attacks glabrous skin. (*d*) *Trichophyton rubrum*, the most common cause of chronic dermatophytosis of the hands, feet, nails, and glabrous skin.

* Received April 9, 1959, from the State University of Iowa, College of Pharmacy, Iowa City.

Abstracted from a dissertation submitted by Dale H. Cronk to the Graduate College of the State University of Iowa in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

† Present address: Northeast Louisiana State College, School of Pharmacy, Monroe.

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EXPERIMENTAL

Synthesis.—The esters and the anilide of sorbic acid[1] were prepared in the usual manner by the action of sorbyl chloride on certain phenolic compounds and aniline. The morpholine and isobutylamine salts were prepared by reacting sorbic acid with the amines. Table I shows the derivatives prepared along with their melting points, yields, and analytical data.

Antimycotic. *Method A.*—The sorbic acid derivatives were tested for antimycotic activity against *Microsporum canis*, *M. gypseum*, *M. audouini*, and *Trichophyton rubrum*. Spore suspensions for inoculation of the plates were prepared by removing pure colony growths from an agar slant and placing them in a 2-oz. wide-mouth bottle. Small pieces of glass rod and 20 ml. of water for injection were added and the bottle was shaken vigorously to disperse the spores. The agar plates were then inoculated with 0.5 ml. of this spore suspension.

The first series of plates were prepared by adding 20 ml. of the following medium, pH 6.2, to each plate: Glucose, 10.0 Gm.; Peptone, 2.0 Gm.; KH_2PO_4, 0.5 Gm.; $MgSO_4$, 0.5 Gm.; Agar, 15.0 Gm.; and Water, *q. s.* 100.0 ml. Two plates per organism per compound were used.

The test compounds, with the exception of morpholine sorbate, were dissolved in acetone in 5% concentrations. Water was used to dissolve morpholine sorbate since it was not soluble to the extent of 5% in acetone. Four drops of the solutions were placed on 12.7-mm. paper disks which were placed in the center of the inoculated plates. The zones of inhibition in mm. were measured after the plates had been incubated for fourteen days at room temperature. These data are recorded in Table II.

Another modification was that the seed cultures were grown in Petri dishes. When the colony had nearly covered the agar surface, 5-mm. squares were cut from an active growth area and transferred to the centers of freshly prepared agar plates. Four disks, impregnated with a compound as described above, were placed equidistant from each other at the periphery of the agar bed. The plates were then incubated at room temperature for fourteen days, after which the total colony growth was meas-

[1] Supplied through the courtesy of Carbide and Carbon Chemicals Co., Division of Union Carbide Corporation.

TABLE I.—ESTERS, ANILID, AND AMINE SALTS OF SORBIC ACID

No.	Ester or Amine Component	Yield, %	M. p., °C.	C, Calcd.	C, Found	H, Calcd.	H, Found	N, Calcd.	N, Found
1	*p*-Chlorophenol	53	64–66	64.86	64.97	4.95	5.21	..	..
2	2,4-Dichlorophenol	65	73–75	56.25	56.09	3.90	4.07	..	..
3	2,4,5-Trichlorophenol	16	100–102	49.65	50.10	3.10	3.28	..	..
4	*p-tert* Butylphenol	36	56–58	78.68	78.49	8.19	7.93	..	..
5	Methyl *p*-hydroxy benzoate	70	91–93	68.29	67.87	5.69	5.41	..	..
6	Hexylresorcinol	5	95–97	75.00	75.86	8.33	8.62	..	..
7	*o*-Hydroxyphenol	28	120–123	70.58	71.13	5.88	6.29	..	..
8	Chlorothymol	34	Viscous liquid	69.06	67.58	6.83	6.94	..	..
9	*o*-Chloro-*m*-cresol	65	49–51	61.10	66.11	5.50	5.56	..	..
10	Aniline	49	150–153	77.00	77.65	6.95	6.76	7.48	7.19
11	N-Isobutylamine	79	[a]	64.86	64.84	10.27	10.06	7.56	7.33
12	Morpholine	96	131–136 with decompn.	60.30	60.75	8.54	8.69	7.03	7.11

[a] No definite m. p., gradually liquefied from 80–90°.

TABLE II.—ZONES OF INHIBITION IN MM., COMPOUNDS AT 5% W/V CONCENTRATION[a]

Compound No.	Organism: *M. canis*	*M. gypseum*	*M. audouini*	*T. rubrum*
1	19	21	19	17
2	21	18	27	19
3	19	16	30	23
4	17	14	16	19
5	0	0	0	0
6	0	17	20	19
7	21	24	26	31
8	50	40	83	54
9	20	18	23	24
10	0	20	16	15
11	18	14	29	29
12	23	0	20	35
Sorbic acid	33	33	41	37

[a] Average of four measurements from disk to edge of growth.

ured in mm. An average of four diameter measurements was recorded. See Table III for these data.

TABLE III.—WIDTH OF COLONY GROWTH IN MM. DIAMETER, COMPOUNDS AT 5% W/V CONCENTRATION

Compound No.	Organism: *M. canis*	*M. gypseum*	*M. audouini*	*T. rubrum*
1	38	59	48	44
2	66	64	56	54
3	57	65	[a]	55
4	60	56	52	48
5	55	54	[a]	59
6	65	59	53	42
7	69	64	[a]	48
8	36	51	21	37
9	61	54	48	50
10	62	65	57	65
11	59	53	48	40
12	59	54	46	47
Sorbic Acid	38	65	32	36

[a] Plate contaminated.
For identification of compounds see Table I.

Method B. This method consisted of streaking 0.5 ml. of spore suspension over the surface of a Sabouraud's agar plate. Solutions of the compounds were incorporated into hydrophilic ointment in 0.3 mole/Kg. concentration based on the sorbate ion content of the molecule. The ointments were placed in collapsible tubes having an orifice about 2 mm. in diameter. Strips of the ointments, 5 cm. long and 2 cm. apart, were placed on the inoculated plates. Zones of inhibition were determined after incubating the plates for nine days at room temperature. The zones were recorded in square mm. They were determined by placing graph paper, divided into 6.3-mm. squares, under each plate and counting the number of squares in the clear areas. These results are shown in Table IV.

TABLE IV.—ZONES OF INHIBITION IN NUMBER OF SQUARE MM. OF AREA, COMPOUNDS TESTED AT 0.3 MOLE SORBATE/KG. OF BASE

Compound No.	Organism: *M. canis*	*M. gypseum*	*M. audouini*	*T. rubrum*
1	238	647	834	540
2	66	476	687	437
3	0	0	0	250
4	0	304	302	449
5	0	0	0	222
6	132	159	488	357
7	331	953	1,322	913
8	1,255	1,322	1,759	1,170
9	699	1,026	1,326	993
10	238	476	461	647
11	1,255	1,945	1,957	1,719
12	1,032	1,771	1,957	1,532
Sorbic Acid	913	1,429	2,461	1,191

DISCUSSION

The results of method A as shown in Tables II and III indicate that only chlorothymol sorbate was more effective than sorbic acid against the four organisms used. When method B was employed, the activity of the compounds was generally greater with chlorothymol sorbate, *p*-chloro-*m*-cresyl sorbate, isobutylamine sorbate, and morpholine sorbate exhibiting the best activity. The difference in the activities shown in methods A and B could be due to the fact that the ointments were prepared on the basis of moles of sorbate ion per 100 Gm. of ointment rather than the w/v percentages, based on the entire compound used in preparing the solutions; hence, more drug was present to inhibit organism growth.

Also, the hydrophilic base may have aided in the dispersion of the drugs through the agar medium which is also aqueous. This factor might be especially true in the case of the water-soluble isobutylamine and morpholine sorbates.

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(1) Rigler, N. E., and Greathouse, G. A., *Am. J. Botany*, **27**, 701(1940).
(2) Geigy, J. R., Swiss pat. 257,648, May 2, 1948; *Chem. Abstr.*, **44**, 3524d(1950).

Binding of Drugs by Plastics I*

Interaction of Bacteriostatic Agents With Plastic Syringes

By E. MARCUS, H. K. KIM, and J. AUTIAN

A preliminary study was undertaken to ascertain if plastic syringes would bind certain bacteriostatic agents having acidic hydrogens. Three types of plastic syringes (nylon, polyethylene, and polystyrene) were included in the investigation. The results indicated that only the nylon syringe had any effect on the agents studied. Binding occurred within a relatively short contact period and was temperature dependent.

IN THE PAST SEVERAL YEARS, in this country and even more in Europe, more and more plastic appliances and devices have been introduced into medical and pharmaceutical practice. These plastic materials are replacing glass, rubber, and metals in such devices as tubings, containers, syringes, etc.

Unfortunately, the wide acceptance of plastics in other fields has created the illusion that plastics are inert and safe for any and all purposes. Some of this thinking has diffused into the medical and pharmaceutical field. There is at present, however, some concern by responsible representatives of the medical profession, pharmaceutical industry, plastic industry, and governmental agencies that certain plastic materials may be harmful, depending upon their use.

Part of the difficulty of recognizing that plastics may create some unfortunate problems with medicinal products is that many times information on a sample of plastic such as polyethylene, polystyrene, etc., will not always apply to another plastic with the same generic name. This may be understood if one considers that numerous formulations are available for the same generically named plastic. For example, one plastic manufacturer states that over two hundred formulations are used to produce polyvinyl chloride tubing.

Literature (1–5) contains much information concerning the acute and chronic toxicity of polymers and various additives upon animals. Less information (6) is available on chronic toxicity in humans. Unfortunately, only meager information is found on the effect of the plastic on a drug system or the effect of the drug system on the plastic.

One of the chief problems encountered with polyethylene is that it is pervious to gases and volatile substances (7). For example, volatile oils in certain pharmaceutical and cosmetic products have penetrated through the walls of the container, causing a change in aroma of the product (8). Permeation of air through the walls of the polyethylene containers has produced a change in color and taste of tetracycline suspension (8). Emulsion stability has been noted to decrease on storage in a plastic container as compared to storage in a glass container (9). Certain oils, such as mineral oil, myrrh, and Nigerian balsam deformed polyethylene bottles over a period of time (8). Sprays consisting of a combination of phenylephrine hydrochloride and thenylpyramine hydrochloride produced a brown precipitate when stored in polyethylene bottles. Clear, colorless polystyrene containers developed a cloudy appearance when exposed to certain fixed oils for several days (9).

Aqueous solutions of pentylenetetrazol have exerted a solvent action on several types of plastics (10). Various other parenteral products when exposed to polyvinyl chloride tubings were found to release acidic constituents to the solution (9). In certain instances, the plastic tubings caused a color change in the parenteral product. Leaching occurred with

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several types of plastic tubings, indicating that one or more of the additives in the tubing were released to the solution, depending upon the drug system exposed.

In the evaluation of certain types of plastic disposable barrels (11), it was found that paraldehyde injection dissolved polystyrene barrels within five hours. Dimercaprol injection produced an etching and clouding on the surface of polystyrene barrels, but did not affect polyethylene or nylon barrels (11). A parenteral product containing diethyl carbonate as a solvent immediately dissolved a plastic hub of a disposable hypodermic needle (12).

The above review indicates that incompatibilities may occur which are easily discernible. It is also obvious that when a visible physical change has occurred in a drug system or the plastic, the medical practitioner will certainly refrain from the use of that medication. If the incompatibility is not so obvious, then the practitioner or nurse will not be alerted as to the consequence of the medication.

Certainly, no one will question the importance of leaching of a substance from the plastic device into an injectable product; but of equal importance to the clinician is the realization that the drug product contains the full strength of the prescribed medication after contact with plastic devices such as tubings or syringes.

From a review of the chemistry of a number of polymers utilized in plastics, the authors speculated that certain polymers could conceivably bind a medicinal agent. Furthermore, it was felt that those polymers containing charged negative centers, such as carbonyl groups, could react with proton donating agents forming an intermolecular complex. In the past few years, a number of such intermolecular reactions have been reported for macromolecules and those chemical agents having acidic hydrogens (13–18).

In order to ascertain if certain types of plastics would appreciably absorb or bind certain medicinal agents, this preliminary study was initiated. The experiment was designed to include three types of plastic barrels previously employed (11) in an incompatibility study. The three plastic barrels were nylon, polyethylene, and polystyrene.

EXPERIMENTAL

Apparatus and Reagents.—Beckman DU spectrophotometer; Beckman pH meter, model G; and constant temperature ovens. Parahydroxybenzoic acid, Eastman Organic Chemicals; methylparaben U. S. P. Heyden Chemical Corp.; propylparaben U. S. P., Heyden Chemical Corp.; sorbic acid, Eastman Organic Chemicals; phenol (C. P.), Allied Chemical & Dye Corp.; and 4-chloro-3-methylphenol, Eastman Organic Chemicals.

Plastic syringes (nylon, polyethylene, and polystyrene) supplied through Becton, Dickinson & Co., Rutherford, N. J.

2,6-Dibromoquinone chloroimide reagent solution: 2,6-Dibromoquinone chloroimide, Eastman Organic Chemicals (80 mg. of reagent dissolved in 25 ml. of acetone-free ethyl alcohol). *Buffer solution, pH 8.3:* Boric acid, 12.369 Gm.; potassium chloride, 14.911 Gm.; sodium hydroxide, 1.600 Gm.; and distilled water to make, 1000 ml. (When 5 ml. of 1% sodium hydroxide is added to 10 ml. buffer solution, a pH of 9.8 is obtained.)

Methods of Analysis.—Each bacteriostatic agent was recrystallized twice according to standard chemical procedures and the melting point determined to insure a relatively pure compound.

Utilizing the procedure suggested by Singer and Stern (19), a colorimetric method of analysis was employed for the determination of phenol in aqueous solutions.

Essentially, the procedure involves the production of a color by the addition of 2,6-dibromoquinone chloroimide reagent solution to phenol in a 1% sodium hydroxide solution. The solution is then adjusted to a pH of 9.8 with boric acid-sodium hydroxide buffer system. After fifteen minutes, the absorbance of the solution is measured at 600 mμ in a spectrophotometer. In all instances, the solution to be assayed is adjusted to contain 8 μg. or less of phenol.

The other five agents were assayed by normal spectrophotometric methods of measuring the absorbance of the solutions in the ultraviolet region at the wavelength giving maximum absorption. For each agent the maximum absorption and concentration are included in Table I. Each of the agents conformed with the Beer-Lambert law within the range of concentrations employed.

TABLE I.—WAVELENGTH OF MAXIMUM ABSORPTION AND CONCENTRATION USED IN CELL

Bacteriostatic Agent	Wavelength, mμ	Concn., mg./100 ml.
Parahydroxybenzoic acid	250	0.8
Methylparaben	256	0.8
Propylparaben	256	0.8
Sorbic acid	256	0.8
4-Chloro-3-methylphenol	280	4.0

Binding as a Function of Concentration and Temperature.—Only the barrels of each type of syringe were employed in this study. The metal canulas were removed from the syringes and the tips at the junction of the canula and the barrel were fused by the application of heat. All of the barrels in the series were of 2-ml. capacity.

Four concentrations (except for phenol) for each agent were prepared using double distilled water as the solvent. To dissolve the propylparaben and the sorbic acid, it was first necessary to dissolve these two agents in a small quantity of alcohol. Each solution was then placed into each type of plastic barrel and stoppered with a suitable

plug. The plugs were composed of rubber stoppers encased in a strip of Parafilm[1] and tightly screwed into the open end of the barrel to minimize vaporization of the solution. It was found that even after forty-eight hours at 50°, the volatilization was not significant to markedly affect the assays.

The barrels were then stored at three different temperatures (5, 30, and 50° ± 1°), taking particular care not to have the solutions in contact with the stoppers. For each concentration, triplicate samples were employed as well as a control sample stored in a Pyrex test tube. After forty-eight hours of storage, the barrels were removed and the solutions from the triplicate samples pooled. These pooled samples were then assayed for their specific agents. Results were calculated as the average value for one barrel.

The results of this experiment indicated that only the nylon barrels bound any of the agents studied. For this reason, only the data of the nylon barrels were included in this paper. Tables II–VII include these results calculated as agent bound to the nylon. The data from the tables are plotted for each agent as bound agent *vs.* unbound agent and are shown in Figs. 1–6.

Binding as a Function of Time.—Another experiment was conducted to determine the extent of binding over a period of one week. In this experiment, solutions of the bacteriostatic agents were prepared at their usual pharmaceutical concentrations and placed into each type of barrel as described previously. Enough samples were prepared (in duplicate) to insure an adequate supply for the various determinations over the one week period. The results of this study are included in Table VIII and Fig. 7.

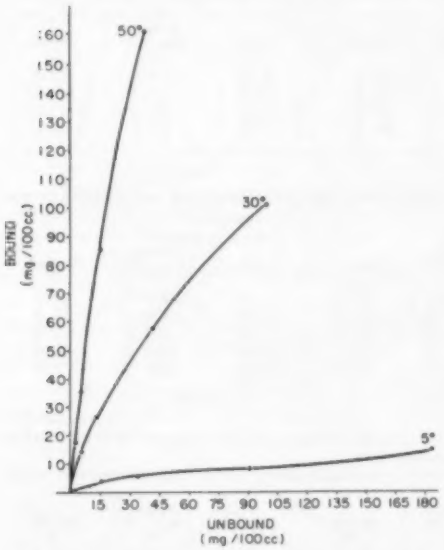

Fig. 1.—Binding of parahydroxybenzoic acid by nylon syringes.

[1] Trademarked product of Marathon Corp., Menasha, Wis.

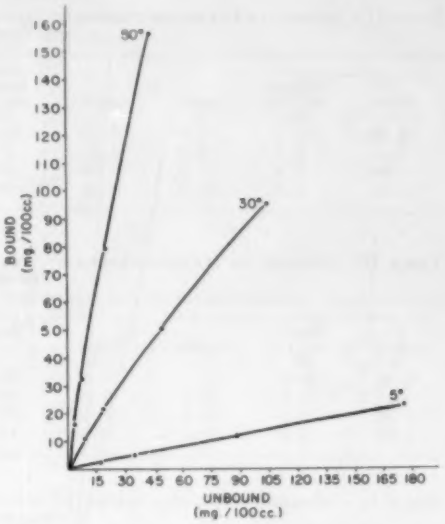

Fig. 2.—Binding of methylparaben by nylon syringes.

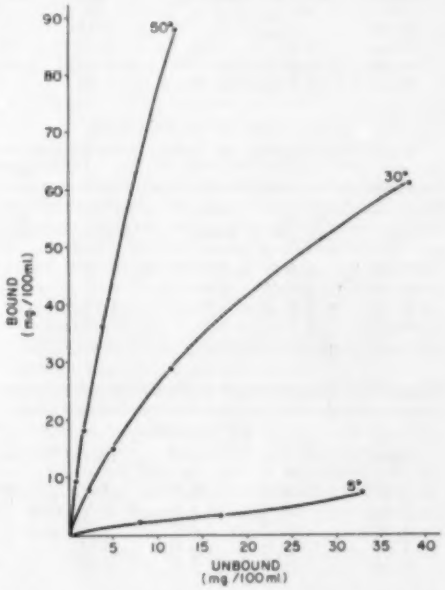

Fig. 3.—Binding of propylparaben by nylon syringes.

Each of the barrels above (after the one-week period) was dried with a clean, lint-free cloth and distilled water was placed into it. After forty-eight hours' storage at room temperature, the solutions were analyzed for the bacteriostatic agent originally present. In all instances, there was desorption occurring. No further study was continued on this desorption experiment.

TABLE II.—BINDING OF PARAHYDROXYBENZOIC ACID BY NYLON BARRELS AS A FUNCTION OF CONCENTRATION AND TEMPERATURE

5°C.			30°C.			50°C.		
Concn., %	Bound, mg./100 ml.	Bound, %	Concn., %	Bound, mg./100 ml.	Bound, %	Concn., %	Bound, mg./100 ml.	Bound, %
0.20	15.1	7.6	0.20	100.7	50.4	0.20	161.5	80.8
0.10	8.6	8.6	0.10	57.2	57.2	0.10	84.9	84.9
0.04	6.9	17.3	0.04	26.7	66.8	0.04	34.7	86.8
0.02	4.2	21.0	0.02	14.2	71.0	0.02	17.4	87.0

TABLE III.—BINDING OF METHYLPARABEN BY NYLON BARRELS AS A FUNCTION OF CONCENTRATION AND TEMPERATURE

5°C.			30°C.			50°C.		
Concn., %	Bound, mg./100 ml.	Bound, %	Concn., %	Bound, mg./100 ml.	Bound, %	Concn., %	Bound, mg./100 ml.	Bound, %
0.20	23.3	11.7	0.20	95.4	47.7	0.20	156.2	78.1
0.10	12.5	12.5	0.10	50.0	50.0	0.10	79.2	79.2
0.04	5.0	12.5	0.04	21.5	53.8	0.04	32.3	80.8
0.02	2.3	11.5	0.02	11.0	55.0	0.02	16.1	80.5

TABLE IV.—BINDING OF PROPYLPARABEN BY NYLON BARRELS AS A FUNCTION OF CONCENTRATION AND TEMPERATURE

5°C.			30°C.			50°C.		
Concn., %	Bound, mg./100 ml.	Bound, %	Concn., %	Bound, mg./100 ml.	Bound, %	Concn., %	Bound, mg./100 ml.	Bound, %
0.10	...	...	0.10	61.3	61.3	0.10	88.0	88.0
0.04	7.2	18.0	0.04	28.7	71.8	0.04	36.2	90.5
0.02	3.2	16.0	0.02	14.9	74.5	0.02	18.3	91.5
0.01	2.1	21.0	0.01	7.7	77.0	0.01	9.2	92.0

TABLE V.—BINDING OF SORBIC ACID BY NYLON BARRELS AS A FUNCTION OF CONCENTRATION AND TEMPERATURE

5°C.			30°C.			50°C.		
Concn., %	Bound, mg./100 ml.	Bound, %	Concn., %	Bound, mg./100 ml.	Bound, %	Concn., %	Bound, mg./100 ml.	Bound, %
0.20	9.8	4.9	0.20	53.8	26.9	0.20	113.8	56.9
0.10	7.8	7.8	0.10	33.4	33.4	0.10	65.5	65.5
0.04	2.8	7.0	0.04	15.7	39.3	0.04	26.5	66.3
0.02	1.6	8.0	0.02	8.1	40.5	0.02	13.6	68.0

TABLE VI.—BINDING OF PHENOL BY NYLON BARRELS AS A FUNCTION OF CONCENTRATION AND TEMPERATURE

5°C.			30°C.			50°C.		
Concn., %	Bound, mg./100 ml.	Bound, %	Concn., %	Bound, mg./100 ml.	Bound, %	Concn., %	Bound, mg./100 ml.	Bound, %
5.00	1750.0	35.0	5.00	3000.0	60.0	5.00	3500.0	70.0
2.00	100.0	5.0	2.00	900.0	45.0	2.00	1300.0	65.0
1.25	62.5	5.0	1.25	565.0	45.2	1.25	812.5	65.0
1.00	50.0	5.0	1.00	450.0	45.0	1.00	650.0	65.0
0.50	25.0	5.0	0.50	225.0	45.0	0.50	350.0	70.0

TABLE VII.—BINDING OF 4-CHLORO-3-METHYLPHENOL BY NYLON BARRELS AS A FUNCTION OF CONCENTRATION AND TEMPERATURE

5°C.			30°C.			50°C.		
Concn., %	Bound, mg./100 ml.	Bound, %	Concn., %	Bound, mg./100 ml.	Bound, %	Concn., %	Bound, mg./100 ml.	Bound, %
0.40	240.0	60.0	0.40	346.0	86.5	0.40	379.2	94.8
0.20	65.0	32.5	0.20	170.0	85.0	0.20	187.6	93.8
0.10	25.0	25.0	0.10	85.0	85.0	0.10	93.8	93.8
0.05	11.3	22.6	0.05	42.5	85.0	0.05	46.4	92.8

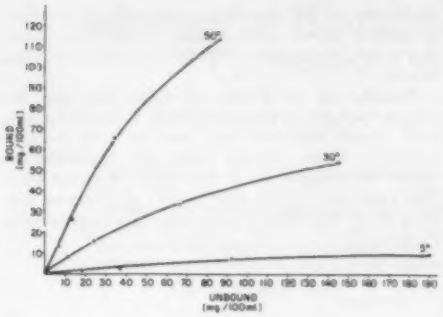

Fig. 4.—Binding of sorbic acid by nylon syringes.

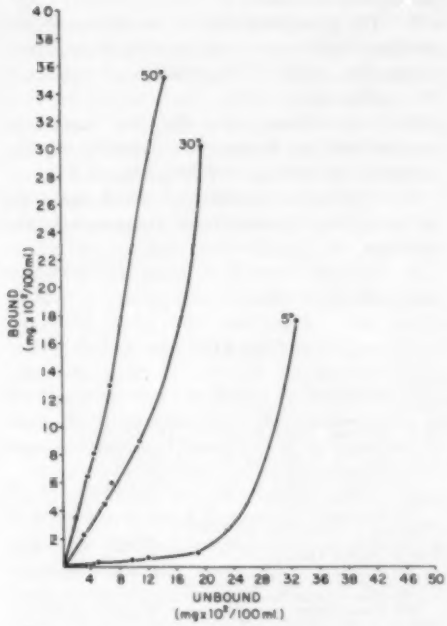

Fig. 5.—Binding of phenol by nylon syringes.

TABLE VIII.—PER CENT OF AGENT BOUND BY THE NYLON BARRELS AT 30°

Drug	Initial	5 hr.	24 hr.	48 hr.	1 wk.
Parahydroxy-benzoic acid (0.2%)	0	14.4	34.6	50.4	78.7
Methylparaben (0.2%)	0	15.3	34.1	47.7	75.5
Propylparaben (0.1%)	0	22.2	44.4	61.3	85.1
Sorbic acid (0.2%)	0	7.1	13.7	26.9	47.0
Phenol (2.0%)	0	16.9	30.7	47.4	60.5
4-Chloro-3-methylphenol (0.2%)	0	8.4	52.6	59.6	85.5

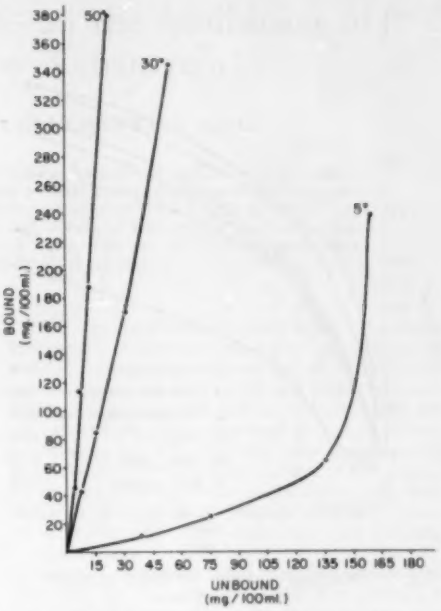

Fig. 6.—Binding of 4-chloro-3-methylphenol by nylon syringes.

RESULTS AND DISCUSSION

Analysis and Equilibrium State.—The analysis of the pooled solutions from three barrels was used in the experiment because it was found that there could be variations in barrel action from one syringe to another. In most instances the difference was small, but occasionally there was a large discrepancy in analytical results from one nylon barrel to another nylon barrel, even though both were distributed by the same source. Also, the pooled samples could be handled more efficiently than the individual samples from one barrel.

Initially, it was felt that forty-eight hours of contact would be sufficient time for equilibrium to be reached, but this did not prove to be the case. Preliminary experimentation on this problem at 30° indicated that a state of equilibrium would not be reached even after several weeks of contact.

Binding as Function of Concentration and Temperature.—In this study the exact mechanism of interaction was not determined, however, it was speculated that the carbonyl groups of the polyamides acted as negative charged centers attracting the proton donating groups of the phenols, the main forces of bonding being through hydrogen bonds. Patel and Kostenbauder (17) have noted this type of interaction when nylon dialysis bags were used in a binding study of certain phenolic preservatives. Pakshver and Mankash (18) postulated that phenols will bind to nylon by a dipole-dipole interaction.

The plots in Figs. 1–6 had a relatively linear relationship at the lower concentration levels. As the concentration levels of the agents (except phenol and 4-chloro-3-methylphenol) were increased the

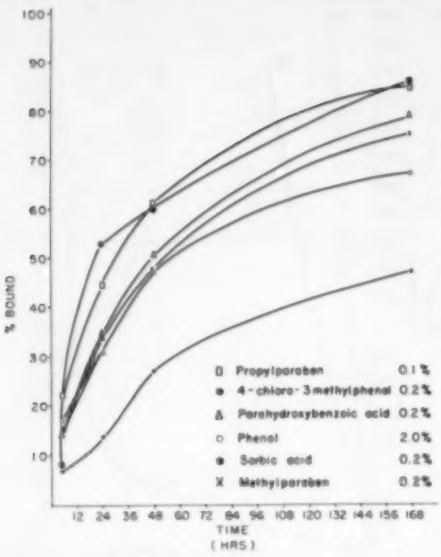

Fig. 7.—Binding of drugs by nylon syringes as a function of time.

slopes of the lines decreased. For a first approximation these curves resemble a Langmuir adsorption isotherm. In dilute solutions, the molecules of the drug are not hindered in approaching the binding sites on the surface and within the nylon, but as the concentration is increased, there is hindrance to the approach of the molecules for the remaining sites.

The above relationship is not seen in Fig. 6 or 7 for the two phenols. In this case, especially observed at 5°, there is an abrupt inflection and increase in the slope (increase in binding) of the line. One explanation for the anomalous results observed for the two phenols is based upon the fact that as the phenolic solution diffuses within the nylon, there is an alteration in the nylon structure (reduction of tensile strength (20)). However, there appears to be a critical concentration level where the solution can literally "break-through" the polymer matrix. Once this has occurred, a great many more sites are available.

It is also obvious from all the figures that as the temperature is increased, the binding phenomenon increases. This probably is explained on the basis that the rate of diffusion is increased and, consequently, more sites in the polymer become available for the drugs to be bound.

Binding as Function of Time.—Table VIII and Fig. 7 show the relationship between time of contact and degree of binding. Even after only five hours of contact at 30°, significant amounts of each bacteriostatic agent were bound. After one week, in nearly all instances, over 60% of the agents were bound.

Significance of Study. Perhaps the real importance of this preliminary study is the realization that plastic materials may not be inert to drug systems. Even though only six bacteriostatic agents were employed in this study, it seems likely that other substances may behave in the same way with nylon or other plastic materials having polar groups.

SUMMARY

1. A study was conducted to determine if certain types of plastic syringes would bind six bacteriostatic agents.

2. The nylon barrel bound to various degrees parahydroxybenzoic acid, methylparaben, propylparaben, sorbic acid, phenol, and 4-chloro-3-methylphenol.

3. Polyethylene and polystyrene barrels did not indicate any tendency to bind the six bacteriostatic agents reported in this study.

4. The degree of binding for each agent was influenced by concentration, temperature, and diffusion.

5. Further research is being conducted on this type of problem.

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Influence of Glutathione on the Inhibition of *Escherichia coli* by Kanamycin*

By ROBERTSON PRATT and YOKO YUZURIHA

The tolerance of *Escherichia coli* ATCC 6522 for kanamycin sulfate was increased from 5 μg./ml. to 15,000 μg./ml. in 63 serial passages through a chemically-defined medium containing increasing concentrations of the antibiotic. Glutathione was shown to interfere with the growth-inhibiting effects of kanamycin on *Escherichia coli* when the kanamycin concentration was 5 μg. or less per ml. and the molecular ratio of kanamycin to GSH was approximately 16 or less.

THE ABILITY of *Escherichia coli* to survive and multiply in the presence of potassium benzyl penicillin may be increased or decreased by the addition of glutathione (GSH). The direction and the magnitude of the change depend on the absolute concentration of penicillin and on the relative concentrations of the antibiotic and the GSH with respect to each other (1). Apparently the complete GSH complement is required to produce the phenomenon, since the constituent moieties of GSH, whether supplied separately or together in the same ratio as in the complete molecule, do not reproduce the effect (2).

We have become interested in extending these observations to other antibiotics. The present report is concerned with the interaction of components in the *E. coli*-GSH-kanamycin system, as expressed through ability of the organism to multiply in a chemically defined nutrient solution containing either or both of the compounds.[1]

Kanamycin was isolated in Japan in 1957 from culture filtrates of *Streptomyces kanamyceticus* (3) and was developed industrially in the United States by the Bristol Laboratories who began to market the sulfate, under the trade name Kantrex, in 1958. Some of the basic and clinical research that preceded marketing of the drug has been collected in a single publication (4).

EXPERIMENTAL

Methods.—*Escherichia coli* ATCC 6522 was maintained as in the work reported previously (1, 2) by daily transfers in Anderson's chemically defined medium as modified by Cowie, *et al.* (5). Inocula for experiments were taken from cultures incubated at 37° for eighteen hours. Organisms were centrifuged and washed three times in sterile distilled water and finally were resuspended to give a transmission of from 46 to 48%, as determined in Pyrex tubes (18 mm., i. d.) in a Lumetron, Model 402 EF, equipped with a neutral filter. One milliliter of this suspension inoculated into 14 ml. of medium provided a suspension with an initial optical density of 0.02 to 0.025 and an initial viable count of approximately 200 × 10^6 organisms per ml. Phosphate buffer was used to adjust culture solutions to pH 6.8. Cultures were incubated in inclined roller tubes rotating at a rate of 5 r. p. m.

Solutions of glutathione and of kanamycin were sterilized by filtration through sintered glass. All other components of the medium were sterilized in an autoclave.

Results.—*Minimum Inhibitory Concentration.*—The MIC for the parent organism was between 5 and 10 μg. of kanamycin per ml. in the medium that was employed. At a concentration of 5 μg./ml., there was a small initial burst of growth during the first five hours, but two hours later the cultures were visually clear and remained so for twenty-four hours (Fig 1). However, growth equal to that of control cultures occurred in forty-eight hours. No growth occurred, even after prolonged incubation, when the organisms were inoculated into media containing 10 μg. of kanamycin per ml.

Resistance.—Upon transfer of organisms that had grown out in forty-eight hours in culture medium containing 5 μg./ml. to fresh solutions containing the same concentration of drug, full growth occurred in twenty-four hours or less. Subculturing these organisms in increasing concentrations of antibiotic yielded, in 63 transfers, a strain that produced full growth in twenty-four hours in medium containing 15,000 μg. kanamycin per ml. Full growth we define as growth equivalent to that of the parent strain (never exposed to the antibiotic) in twenty-four hours in the standard antibiotic-free medium.

Effect of Glutathione.—All experiments with GSH were conducted with the parent strain of *E. coli* which had not been exposed previously to kanamycin. Maximum growth of the organism was not affected by addition of from 0.02 to 200 μg. of GSH/ml. to the antibiotic-free culture medium. The rate of growth during four to five hours was slightly accelerated when the medium contained 20 μg. or 200 μg. of GSH/ml. but not when it contained lesser concentrations.

In culture solutions containing 1 μg. of kanamycin per ml. there was some reduction of growth during the first few hours after inoculation but no significant effect after twenty-four hours (Fig. 1). Supplied at an initial concentration of 2 μg./ml., kanamycin caused substantial retardation of growth for at least seven hours and an appreciable reduc-

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Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

[1] The kanamycin used in this work was kindly provided by the Bristol Laboratories.

tion even at the end of twenty-four hours. However, these effects were eliminated when as little GSH as 0.02 μg./ml. ($6.5 \times 10^{-8} M$) was added to the medium, and growth curves for these cultures and those containing larger amounts of GSH were the same as for antibiotic-free control cultures provided with the corresponding concentrations of additive.

Addition of GSH at a level of 0.2 μg./ml. ($6.5 \times 10^{-7} M$) partially reversed the inhibitory action of kanamycin supplied at a concentration of 3 μg./ml. Lower concentrations of GSH had no effect. As the concentration of GSH was increased to 2.0 and then to 20 μg./ml., there was progressive diminution of the inhibitory action of the antibiotic during the first seven hours of growth, and after twenty-four hours the optical density of cultures initially containing 3 μg. of kanamycin and from 0.2 to 20 μg. of GSH per ml. was equal to that of the control culture (Fig. 2).

At kanamycin levels of 4 μg. and 5 μg./ml., addition of GSH also partially reversed the action of the antibiotic (Fig. 2). No growth occurred, even upon prolonged incubation, in media containing 10 μg. of kanamycin per ml., irrespective of the concentration of GSH.

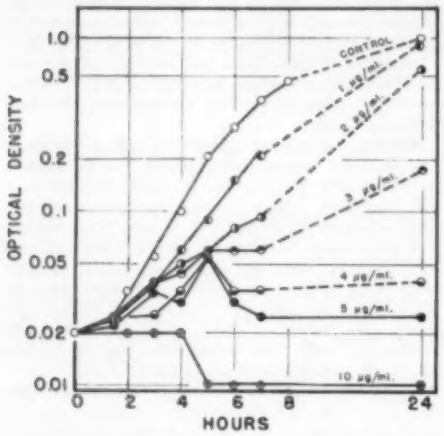

Fig. 1.—Growth of *Escherichia coli* ATCC 6522 in a chemically-defined medium containing the indicated concentrations of kanamycin.

The approximate parallelism of the seven-hour curves for 3, 4, and 5 μg. of kanamycin per ml. (Fig. 2) indicates that a given increase in GSH concentration was accompanied by an approximately equal (percentagewise) reduction in the inhibitory effectiveness of the antibiotic, irrespective of the concentration of the latter. At GSH concentrations above 20 μg./ml., the curves flattened off. The optical density at seven hours for cultures containing 200 μg. of GSH per ml. was not significantly different from that of cultures containing only 20 μg. of additive per ml.

A different situation obtained at twenty-four hours. In cultures containing, respectively, 3, 4, and 5 μg. of kanamycin per ml., a given increase in GSH concentration was accompanied by progressively greater reduction in inhibitory effectiveness of the antibiotic as the concentration of the latter increased. This is indicated by the progressively increasing slopes of the curves for the increasing concentrations of antibiotic (Fig. 2). As was found for the growth at seven hours, a GSH concentration of 0.02 μg./ml. had no effect on the total twenty-four-hour growth of organisms exposed to 3, 4, or 5 μg. of kanamycin per ml., and raising the GSH concentration to 200 μg./ml. had no significant effect beyond that associated with the 20 μg./ml. level.

Calculations of molecular concentrations showed that, as a generalization, when the absolute concentration of kanamycin was in the range 3 to 5 μg./ml. (6.2×10^{-6} to $10.3 \times 10^{-6} M$) and the kanamycin-GSH index (molecular concentration of kanamycin divided by molecular concentration of GSH) was of the order of approximately 16 or less, the effectiveness of the antibiotic against *E. coli* was diminished. This was a purely empirical observation, and we do not mean at this time to suggest that biochemical implications with respect to mechanisms of action of kanamycin can be drawn from it.

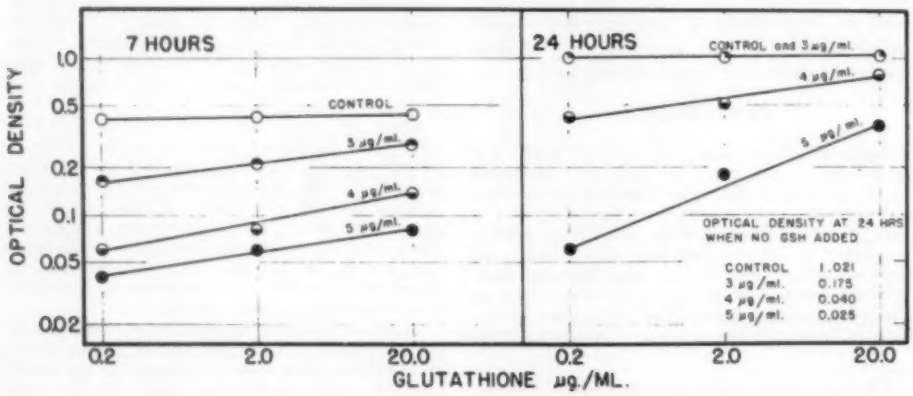

Fig. 2.—The effect of increasing concentrations of glutathione on the inhibitory action of the indicated levels of kanamycin against *Escherichia coli* ATCC 6522.

DISCUSSION

It remains to be determined whether inhibitory effects of GSH on the antibacterial action of kanamycin, similar to those described above, can be demonstrated with other organisms and in other media. Also remaining to be determined is the significance, if any, of the increasing inhibitory effect at twenty-four hours of a given increment of GSH concentration as the concentration of kanamycin was increased from 3 to 5 μg./ml. when no such effect could be observed at seven hours.

However, the observed data suggest interesting speculation concerning their possible clinical significance. Currently available data indicate that in adults a daily dosage regimen of 0.25 Gm. per eight hours can be expected to produce serum concentration of kanamycin ranging at different times from about 2.0 to 12.0 μg./ml. or that on a 0.5 Gm. per twelve-hour schedule a range of about 2.0 to 28.0 μg./ml of serum can be anticipated, depending on the time elapsed since the last previous injection and factors of patient variability (6). The figures are roughly compatible with data showing a range from 1.3 to 14 μg./ml. of serum in infants and children receiving a single intramuscular dose of 5 mg./Kg. (7). Assuming that serum accounts for approximately 60% of the total volume of whole blood (8) and that most, if not all, of the blood-kanamycin is in the serum, the above figures are comparable to whole blood levels ranging from less than 1.0 to about 17 μg./ml.

Blood or serum levels of antibiotics are not important in themselves, in the absence of bacteremia. Since the primary action of most antibiotics is exerted directly on the invading organisms and not indirectly, as for example, through effects on host defense mechanisms, it is the actual concentration of the drug at the sites where the pathogens are that is important in determining clinical effectiveness. But since it is not always possible or, if possible, convenient to determine concentrations at the specific sites of infection, it has become conventional to rely on the blood level as an index of therapeutic concentration. Justifiable as this practice is empirically, it is important to bear in mind that the blood level is simply a symbol and, even though it may bear a definite relationship to the antibiotic concentrations in various other body tissues and fluids, not to confuse it, in absolute terms, with the value for which it is merely an index. The figures cited in the preceding paragraph indicate that at certain periods during treatment with kanamycin the concentration of the antibiotic, as judged by the response of *E. coli*, falls within the range in which action of the drug is subject to interference by GSH. It seems entirely possible that the periods during which such concentrations prevail in other tissues and fluids may be reached sooner and last longer.

Reliable data are available on the GSH content of blood and of a few specific organs, e. g , the eye and the liver, but there seems to be a scarcity of data relating to body tissues or organs in general. However, tissue levels of GSH probably are usually at least as high as the minimum concentration (0.02 to 0.2 μg./ml.) shown above to interfere with the antibacterial action of low concentrations of kanamycin against *E. coli* in a synthetic medium.

The normal GSH content of human blood has been reported to be about 350 μg./ml. (9). In severe mental disease (schizophrenia, manic-depressive psychosis, and involuntional melancholia), the GSH index may be reduced as much as 40% (10). Likewise, "marked reduction in blood GSH" has been observed in ketonemia accompanying diabetes and in diffuse and severe liver disease. Even so, the GSH content of the blood is not likely to fall below 175 to 200 μg./ml. Presumably most of the GSH is in the formed structures (mostly erythrocytes) of the blood (9), but some may also be present in serum.

The inhibitory effect of GSH on the antibacterial activity of low concentrations of kanamycin may merit consideration in planning injection schedules aimed at achieving maximum clinical effectiveness of the drug.

SUMMARY

The effect of kanamycin on the growth of *Escherichia coli* ATCC 6522 in a chemically defined medium, with and without addition of glutathione (GSH), has been studied.

1. The minimum inhibiting concentration of kanamycin in the absence of GSH was between 5 and 10 μg./ml.

2. A 3,000-fold increase in tolerance was developed in 63 serial passages of the organism through increasing concentrations of the drug.

3. GSH in a concentration of 0.02 μg./ml. completely nullified the inhibitory effect of kanamycin supplied in concentrations of 1 or 2 μg./ml.

4. In a concentration of 0.2 μg. or more per ml., GSH partially or completely reversed the antibacterial action of kanamycin provided at 3, 4, or 5 μg./ml. The degree of reversal was a logarithmic function of GSH concentration between 0.2 and 20 μg./ml. and depended on the time at which observations were made.

5. Speculation on a theoretically possible clinical implication of the data is suggested.

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Saponins and Sapogenins IV*

Isolation of Oleanolic Acid from *Sesbania Aegyptica* Pers.

By M. O. FAROOQ, I. P. VARSHNEY, and MOHD. S. Y. KHAN

The seeds of *Sesbania Aegyptica* Pers. have been found to contain two triterpenic sapogenins, one an acid sapogenin and the other a neutral one. The acid genin has been identified as oleanolic acid by a direct comparison of the melting point and the infrared spectra of the methyl ester of the acetate with an authentic sample.

Sesbania Aegyptica Pers., locally known as "Ravasin," is a member of the family *Leguminosae*, sub-family *Papilionatae*, and grows wild throughout the plains of India. Owing to its rapid growth it is being increasingly used as a hedge plant and it is a good cattle feed. The seeds are used in cases of the enlargement of the spleen (1). A number of the plants of the family *Leguminosae* have been found to contain saponins and sapogenins (2–5), but the only record about this plant is that it contains no saponin (6). During our work on the fat content (7) of the seeds of the plant, the presence of a saponin was indicated and therefore its systematic examination for the saponin content was taken up.

The seeds of *Sesbania Aegyptica* Pers. were procured from M/S. N. Cooper & Co., Poona, as well as obtained from the plants grown on the University campus. A quantity of the finely powdered, defatted seeds were extracted with ethanol. The recovery of the solvent left an oily residue which was successively treated with ether, petroleum ether, carbon tetrachloride, and acetone. The residue thereafter was taken up in alcohol and precipitated by addition to a large amount of ether. This precipitation was repeated a number of times. This gave colorless saponin which became a brown syrup on exposure to atmosphere and satisfied all the tests for saponin. The saponin was dissolved in a large amount of water and hydrolyzed with sulfuric acid. The genin obtained was filtered and washed free of the acid. The failure of a number of attempts at crystallization from different solvents suggested the genin to be a mixture and, therefore, it was refluxed with a solution of alcoholic potassium hydroxide and extracted with ether. Evaporation of the ethereal layer gave a neutral genin while the alkaline solution, on treatment with an excess of hydrochloric acid, gave a precipitate of an acid genin. This acid genin gave an acetate which, on crystallization from methanol, had m. p. 261–263°, $[\alpha]_D$ = +72.7. The acetate gave positive response to all the usual tests for a triterpene.

The resemblance of the infrared spectrum of the acetate with the spectra of the acetates of the triterpenic acids of β-amyrin group (methylene bending absorption) was very close and did not resemble at all the infrared spectra of the acetate of the steroidal sapogenins (finger print region) (2, 4).

The deacetylation of the acetate gave an acid genin, m. p. 285–288°, $[\alpha]_D$ = +80. The analysis of the acetate indicated the presence of one hydroxyl and one carboxyl group in a pentacyclic triterpenic compound.

The carboxyl function which was readily detectable in the infrared spectra was fixed by the formation of a methyl ester, m. p. 195–198°, $[\alpha]_D$ = +76. The fact that it was obtained with diazomethane and not with methanolic hydrochloric acid, coupled with the difficulty of its hydrolysis, suggested the attachment of the carboxyl group to a tertiary carbon atom (i. e., to C-17). The methylation of the acetate with diazomethane gave an acetyl methyl ester, m. p. 217–219°, $[\alpha]_D$ = +72.7. The genin and all its derivatives showed unsaturation with tetranitromethane.

The genin on oxidation with chromic acid gave a ketone which gave a positive Zimmermann test (8) showing the position of the carbonyl group at C-3. The ketone gave a 2,4-dinitrophenylhydrazone without difficulty.

The relation with the β-amyrin group was further established by the examination of the U. V. spectrum of the product of oxidation of the acetate with selenium dioxide. The product obtained on oxidation could not be crystallized and showed the characteristic triple ultraviolet absorption maxima ($\lambda_{max.}^{EtOH}$ 241, 249, 255 mμ) of a diene.

By analogy with all the acids of the β-amyrin group the position of one of the hydroxyl groups has been assumed to be at C-3 in ring A, and that of the carboxyl group at C-28 attached to C-17. The comparison of the physical constants of the

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For earlier three parts see (5).

The authors are grateful to Prof. T. R. Govindachari of the Presidency College, Madras, for the infrared spectra.

genin and its derivatives with all the known acids of the β-amyrin group carrying one hydroxyl group indicates the present genin to be identical with oleanolic acid (Table I).

The comparison of the infrared spectra (Fig. 1) and mixed melting point of the acetyl methyl ester of the genin with authentic samples of acetyl methyl oleanolate obtained from *Albizzia lebbek* (2) and *Randia dumetorum* (9) confirmed the identity of the genin as oleanolic acid.

TABLE I.

	Oleanolic Acid m. p.	$[\alpha]_D$	Present Genin m. p.	$[\alpha]_D$
Genin	310	+80	285–288	+80
Acetyl oleanolic acid	268	+74.5	261–263	+72.7
Methyl oleanolate	198–200	+75	195–198	+76
Acetyl methyl oleanolate	219–220	+70	217–219	+72.7

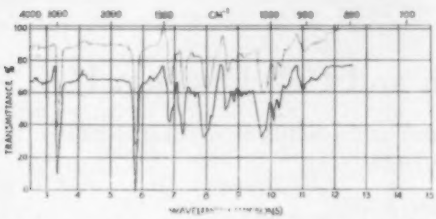

Fig. 1.—Comparison of infrared spectra. Solid line shows present genin acetyl methyl ester. Broken line shows acetyl methyl oleanolate.

EXPERIMENTAL

All the melting points recorded in this paper have been taken on a Kofler hot microscopical stage and are corrected. The infrared spectra have been taken in chloroform solution at the Presidency College, Madras, using a double beam Perkin-Elmer spectrometer Model 137 (Infracord) and interpreted by one of the authors (I. P. V.). The microanalyses recorded here have been done at the Department of Chemical Technology, University of Bombay. The ultraviolet spectra have been measured using a Beckman spectrophotometer Model DU.

Defatting.—Well-powdered seeds (1 Kg.) were exhausted in a Soxhlet extractor with light petroleum ether (40–60°). The recovery of the solvent left a greenish oil (55 Gm.). The exhausted seeds were dried before further operation.

Extraction.—The defatted seed powder was exhausted with 95% alcohol in a Soxhlet extractor and the solvent recovered leaving a brown syrupy liquid. The residual syrup was dissolved in ethanol and filtered. The alcohol was recovered and the solid residue extracted with ether, petroleum ether, carbon tetrachloride, and acetone, successively. This gave a brown syrupy mass, which was dissolved in a little alcohol and added to a large amount of ether, which precipitated the saponin. This process of dissolution in alcohol and precipitation by ether was repeated three times. It gave colorless saponin which turned to a syrup on exposure to air, and gave all the tests for saponin.

Isolation of Sapogenin.—The brown syrupy mass (25 Gm.) was dissolved in water (6 L.) and hydrolyzed with sulfuric acid (300 Gm.) by heating the solution first on a boiling water bath for an hour and thereafter completing the hydrolysis by boiling the solution for another hour. After about twenty to thirty minutes a precipitate began to appear which went on increasing until the hydrolysis was completed. It was then filtered and washed with water until the filtrate was neutral. It was dried in an air oven at 80°. The dried genin was dissolved in alcohol and decolorized with charcoal. All attempts at crystallization from various solvents proved fruitless.

Separation of Acid and Neutral Genin.—The crude genin (1 Gm.) was heated with alcoholic caustic potash (20 Gm. KOH in 300 cc. of alcohol) for a half-hour and then half the solvent was distilled off. The solution was then diluted with water (2 L.) and extracted three times with ether. The ethereal extracts were combined and washed free of the alkali. It was dried over sodium sulfate (anhydrous) and removal of the ether left a neutral substance in the flask.

The alkaline solution was acidified with hydrochloric acid when it gave a precipitate. This was filtered, washed free of the acid, and dried.

Acetylation.—The acid genin obtained as above was acetylated by treatment with pyridine and acetic anhydride in the cold for eighteen hours. It was poured into ice water and filtered. The precipitate was washed free of the acid and pyridine and crystallized twice from methanol to obtain the product in fine, colorless needles, m. p. 261–263°, $[\alpha]_D^{29}$ = +72.7 (C = 0.165 $CHCl_3$), yield = 500 mg. It gave a positive test with tetranitromethane, $\lambda_{max.}^{CHCl_3}$ 5.8, 5.9, and 8.0μ.

Anal.—Calcd. for $C_{32}H_{50}O_4$: C, 77.11; H, 10.40. Found: C, 77.0; H, 9.8.

Deacetylation.—The acetate (250 mg.) was refluxed for two hours with 15 cc. of 5% methanolic potassium hydroxide. The solution was diluted with 200 cc. water and left overnight at room temperature. It did not yield any crystalline potassium salt. The solution was acidified with hydrochloric acid and the precipitate formed was washed free of the acid and crystallized from methanol, m. p. 285–288°, $[\alpha]_D^{26}$ = +80 (C = 0.100 $CHCl_3$). It gave a positive test with tetranitromethane.

Selenium Dioxide Oxidation.—Acetate (100 mg.) in 15 cc. acetic acid was heated under reflux with 100 mg. freshly sublimed selenium dioxide for two hours. It was poured into water and extracted with ether. It could not be crystallized. $\lambda_{max.}^{EtOH}$ = 241, 249, 255 mμ.

Methylation.—The acid genin (200 mg.) was dissolved in 200 cc. ether and an excess of ethereal solution of diazomethane added. It was left overnight, the excess of diazomethane was removed on a water bath, and the product was crystallized from methanol, m. p. 195–198°, $[\alpha]_D^{28}$ = +76 (C = 0.098 $CHCl_3$). The substance gave a positive test with tetranitromethane.

Anal.—Calcd. for $C_{31}H_{50}O_3$: C, 79.1; H, 10.6. Found: C, 78.2; H, 10.8.

Demethylation.—The methyl ester (100 mg.) was refluxed with 100 cc. of 5% methanolic caustic potash for half an hour. The starting material (80%) was recovered unchanged and only 20% of the ester was demethylated. The genin obtained was confirmed by melting and mixed melting points.

Acetyl Methyl Ester.—The acetate (100 mg.) was dissolved in ether and an excess of a solution of a diazomethane added to it. After a contact of twenty hours ether was removed and the residue crystallized from methanol, m. p. 217–219°, $[\alpha]_D^{25} = +72.7$ (C = 0.165 $CHCl_3$). It gave a yellow color with tetranitromethane. $\lambda_{max.}^{CHCl_3} = 5.8, 8.0\ \mu$.

Anal.—Calcd. for $C_{32}H_{52}O_4$: C, 77.29; H, 10.22. Found: C, 77.3; H, 9.7.

Oxidation of the Genin.—A solution of chromic acid (100 mg.) in 15 cc. of 80% acetic acid was added to 100 mg. of the genin dissolved in 20 cc. acetic acid. After leaving it for half an hour at room temperature, 100 cc. alcohol was added to destroy the excess chromic acid. The solution was left for fifteen minutes and then the alcohol was distilled off under reduced pressure. The residue was dissolved in aqueous alcohol and extracted with ether. The ethereal solution was washed well with water and evaporated to dryness. The product crystallized from methanol as fine needles, m. p. 156–160°. It gave a positive Zimmermann test.

2,4-Dinitrophenylhydrazone.—The ketone (50 mg.) was dissolved in 10 cc. alcohol and heated on a water bath. It was then mixed with a solution of 50 mg. 2,4-dinitrophenylhydrazine in 10 cc. alcohol and 0.4 cc. hydrochloric acid. It was left at room temperature for five minutes when a precipitate was formed. It was kept overnight, filtered, washed, and recrystallized from ethanol, m. p. 251–255°.

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Studies on Cell Growth and Cell Division I*

A Temperature-Controlled System for Inducing Synchronous Cell Division

By KWAN-HUA LEE

A temperature-controlled system for inducing cell growth and synchronous cell division in *Tetrahymena pyriformis* is described. By using this device, it is possible to dissociate cell growth and cell division into two distinct processes.

RECENTLY, Scherbaum and Zeuthen (1) have demonstrated that when proteose-peptone cultures of the ciliate protozoon *Tetrahymena pyriformis* GL were incubated at alternate half-hour periods at 28° (optimum for growth) and 34° (sublethal) for six or seven cycles, the size of the ciliates was increased to about three times larger than the normal cells without any cell division taking place. When these heat-treated cells were brought back to 28°, either in a nutrient medium or in a nutrient-free buffer solution (2), they went into cell division synchrony for three times before normal random cell division was resumed. In the nutrient medium, both cell division and cell growth took place while in the nutrient-free medium only cell division was possible. This technique dissociates cell growth and cell division into two distinct processes and thus provides biological material for the studies on selective activity of drugs on either process without any interference of the other process.

The technique of inducing synchronous cell division has since been applied to other strains of *Tetrahymena pyriformis* (3) and many other forms of unicellular organisms (4). In this paper, a practical temperature-controlled system for inducing cell growth and cell division is described. The principle applied in the design has many obvious advantages and it can also be adjusted to cycle at any other temperature levels at specified rate and intervals.

* Received April 8, 1959, from the School of Pharmacy, University of California Medical Center, San Francisco 22.

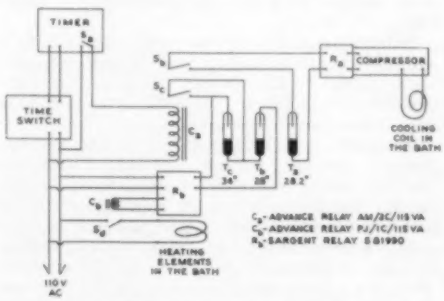
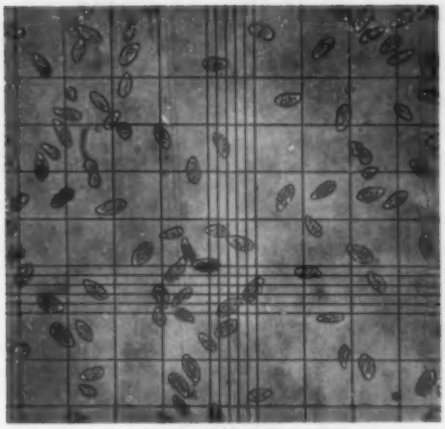
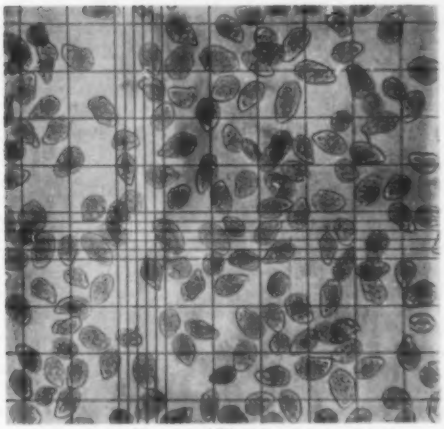


Fig. 1.—Wiring diagram of the water bath.



(*A*)



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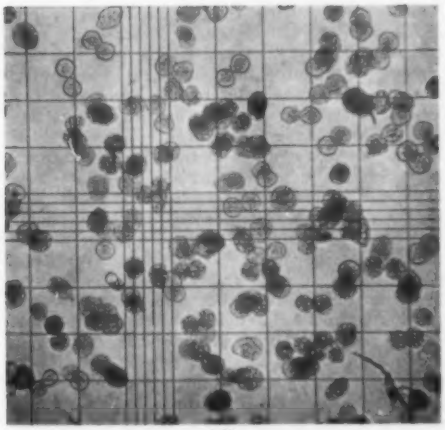

(*C*)

Fig. 2.—(*A*) Normal cells, (*B*) heat-treated cells, (*C*) synchronous dividing cells.

The wiring diagram of this system is shown in Fig. 1. The induced cell growth and synchronous cell division in *Tetrahymena pyriformis* is shown in Fig. 2. The following is a brief account of its operation.

The timer, a clock motor (Model 660, Automat. Elec. Mfg. Co.) is used to close or open switch Sa at preset time intervals. In the first half of the time cycle when switch Sa is on, coil Ca is energized and closes switches Sb and Sc. If the temperature is higher than 28.2°, thermostat (mercury thermoregulator, Aminco) Ta is in contact and energizes the coil in relay Ra to set on the compressor motor until the temperature is brought down just below 28.2°. When the temperature is lower than 28°, thermostat Tb is open and coil Cb is energized to close switch Sd and the heating elements are on until the temperature reaches 28°. From now on the room temperature (lower than 28°) and the heating unit operated by thermostat Tb, control the bath at 28°. The compressor motor is used continuously and not intermittently for only twelve minutes in each period of one hour.

In the second half of the time cycle when the timer opens switch Sa, thermostat Tc is in operation. As shown in the diagram, Tc shares essentially the same circuit as Tb except that Tc is set at 34°. In the present case, Tc is used to raise the temperature from 28° to 34° and maintains that temperature as long as switch Sa is in open position.

To adjust the rates of heating and cooling between 28° and 34° according to the empirical findings of Scherbaum and Zeuthen (1), it is best to first adjust the quantity of water in the bath so that it cools from 34° to 28° in twelve minutes. For warming up, one can easily estimate the wattage of the heating elements required so that the temperature can be raised to 34° from 28° in eight minutes.

The surface area of our bath is 12 × 28 inches. The amount of water used is about 30 L. The compressor used is a Westinghouse Type E T-A Model and the heating elements are two copper-sheathed heaters of one kilowatt each.

It will be more convenient if a time switch (Type 471, G. E.) is connected in the circuit so that it can set the timer switch on at midnight so that in the morning the heat-treated cells are ready for the studies on cell growth or cell division.

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Studies on Cell Growth and Cell Division II*

Selective Activity of Chloramphenicol and Azaserine on Cell Growth and Cell Division

By KWAN-HUA LEE, YOKO OKANO YUZURIHA, and JOHN J. EILER

A simplified practical procedure for the study of drug action on cell growth and cell division as two distinct processes is described. To illustrate one of the many uses of this system, the selective activity of two antibiotics, chloramphenicol and azaserine on cell growth and cell division is demonstrated.

IN MASS CULTURES of unicellular organisms, cells grow and divide at random. The conventional methods, using mass cultures in the study of drug actions, have provided valid estimates of the overall biological activity of drugs, but have not permitted a clear-cut differentiation between effects of drugs on cell growth and cell division.

Recently, Scherbaum and Zeuthen (1) developed a controlled-temperature shift technique which permits the study of cell growth and cell division as separate processes in mass culture of *Tetrahymena pyriformis*. When a culture of *Tetrahymena pyriformis* GL is exposed to alternate half-hour periods at 28° (optimum for growth) and 34° (sublethal) for seven or eight cycles, the cells continue to grow in size up to about three times the size of the initial normal cells, with little or no increase in cell number. During this temperature-cycling period, it is possible to study the effect of drugs on cell growth alone. When these overgrown heat-treated cells are maintained at 28°, either in a nutrient medium or in a nutrient-free salt buffer solution (2), they go into cell division synchrony three times before normal random cell division is resumed. In the nutrient medium, both cell division and cell growth take place, while in the buffer solution only cell division is observed. During this cell-dividing period, it is possible to study the effect of drugs on cell division.

In the first paper of this series (3), there was presented the design of an automatic temperature-control system which duplicates nicely the conditions established by Scherbaum and Zeuthen (1) as being necessary to the separation of the two phases of cell regeneration.

In this paper, a simplified practical procedure to test the effect of drugs on either the growth or the division process, or on both, in *Tetrahymena pyriformis* is presented.

To illustrate one of the uses of this procedure, the results of the study of the selective activity of two antibiotics, chloramphenicol and azaserine,[1] are demonstrated. Chloramphenicol, in appropriate concentrations, inhibits cell growth but not cell division, while azaserine inhibits cell division, but not cell growth.

METHODS

Culture—*Tetrahymena pyriformis* GL was obtained through the courtesy of Professor G. W. Kidder and maintained in a proteose-peptone (Difco), liver extract fraction L (Wilson Laboratories) and salt medium (1). Stock cultures were transferred daily by means of an inoculating loop to 6 ml. of medium in test tubes (15 × 150 mm.) in a slanted position and maintained at 28°. For the experimental culture, 200 ml. of medium in a 500-ml. Erlenmeyer flask was inoculated with 0.3 ml. of a two-day old culture (containing about 10^9 cells) and maintained at 28°. At the end of eighteen hours, the culture contained about 35,000 cells/ml. and was used in the heat treatment as described below.

Cell Growth.—For the study of the effects of the drugs on cell growth, 20-ml. portions of the experimental culture were transferred into 250-ml. Erlenmeyer flasks. Samples to serve as initial controls for cell count and protein content were taken at this time. The drug under study was first dissolved in approximately 0.1 ml. of pH 6.8 salt buffer solution (2) and then added to some of the flasks. These flasks together with three controls were then clamped on the shaking device which has a horizontal movement of 4 cm. displacement at fifty strokes per minute in the temperature-controlled water bath (3) and subjected for seven cycles of heat treatment. Each cycle consisted of half an hour at 34° followed by half an hour at 28°. At the end of the seventh cycle, the growth of the cells in the flask was stopped by the addition of two drops of Bouin's formaldehyde reagent (4) or by immersing the flasks in a boiling water bath for not more than fifteen seconds. Prolonged heating or too much formaldehyde reagent added caused difficulties in solubilization of the cells in biuret reagent. The formaldehyde-treated or heat-killed cells were transferred to 50-ml. conical centrifuge tubes and centrifuged. The cells were washed twice with buffer solution and then analyzed for protein content by the use of the biuret method (5). When the amount of cell protein analyzed was less than 4 mg., removal of lipid by repeated extraction with alcohol-

* Recieved June 9, 1959, from the School of Pharmacy, University of California Medical Center, San Francisco 22.

[1] Graciously supplied by Dr. E. L. Wittle of Parke, Davis Research Laboratories, Detroit, Mich.

ether mixture (1:1 v/v) or trichloroacetic acid-alcohol mixture (6) is not necessary.

For the study of cell growth in the absence of drug, a series of flasks, each containing 20 ml. of experimental culture, were subjected to the temperature cycling as described above. Flasks were removed from the bath at the end of each cycle and at the end of the period of synchronous cell division, as indicated in Fig. 1, for cell protein analysis and cell counting.

Cell Division.—For cell division studies in the absence of nutrient, the cells in one of the three flasks used as controls in the growth studies, at the end of the last cycle, were washed with buffer solution in the hand centrifuge. The washed cells were then suspended in 10 ml. of the buffer and initial samples for cell count were taken. One-ml. portions of the cell suspension were transferred to a series of 50-ml. centrifuge tubes containing one ml. of buffer with the appropriate concentrations of drug and placed in the water bath at 28°. The time required for preparing these cells for the division studies was less than five minutes. After five and one-half hours, 0.6 ml. of formaldehyde reagent was added to each tube and the cells were counted in a Sedgwick-Rafter counting chamber, using a Whipple ocular micrometer disk (7).

For the studies on the effect of drug on cell division in the presence of nutrient, a series of flasks, each containing 20 ml. of experimental culture, were subjected to temperature cycling as described. At the end of the last cycle, samples were taken for cell count and the drug in appropriate concentrations was added to some of the flasks and the incubation was continued at 28°. At the end of five and one-half hours, samples were taken for cell count.

RESULTS

Cell Growth and Cell Division.—The results of the study of cell growth and cell division in the absence of drug are presented in Fig. 1. The ordinate expresses the changes in cell protein (growth) and cell count (division) relative to the initial values. The drawings in the figure are to indicate the approximate change in cell size and cell number during the various procedures. The average amount of protein in the initial log phase cell was about 3×10^{-3} μg./cell (with a variation from 2.6 to 3.2×10^{-3} μg./cell). The average initial cell count was 35,000 cells/ml.

In Fig. 1 it should be clear that during the period of temperature cycling, the increase in cell protein was not accompanied by any significant increase in cell number. During this period the protein content of the constant population of cells increased to more than three times that of the initial normal cells. Such an increase is in excellent agreement with other measures of the cell growth during this period, such as dry weights (8) and cell volume (9). It should also be noted that an increase in total protein took place during the period of constant temperature when cell division was permitted to occur in the *presence* of nutrient. Here, over a period of five and one-half hours the protein of the cells increased to double the value at the end of the temperature cycling and to six times that of the initial value. The increase in protein during the period of constant temperature was, of course, accompanied by cell

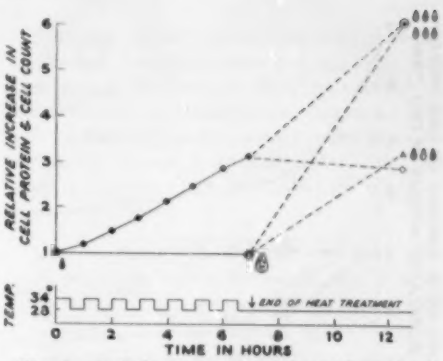

Fig. 1.—Relative change in cell protein and cell count under the various conditions in the absence of drug. The symbols Δ (relative cell count) and ◊ (relative protein) refer to those experiments wherein the cells were transferred to nonnutrient medium after the seven hours of heat treatment in the presence of nutrient. The symbols ○ (relative cell count) and ● (relative protein) refer to those experiments in which the cells were maintained in nutrient. The drawing indicates the increases in size and number of *Tetrahymena* under the several conditions.

division. Interestingly, simple calculations permit the view that during both periods the increases in protein were linearly related to the time spent at 28°, regardless of the occurrence or nonoccurrence of cell division. No increase in protein was observed when cell division was permitted to take place in the *absence* of nutrient. Indeed, there was observed a slight decrease in protein, probably due to catabolic utilization.

The increases in cell number during the period of constant temperature, both in the presence and in the absence of nutrient, were in good agreement with the increases in cell protein noted above. We have observed the three expected (1,2) sets of synchronous division both in the presence and in the absence of nutrient. In the absence of nutrient, during the five and one-half hours at constant temperature, the cell population increased to a value 3.2 times the starting population. Such an increase was made possible by the increase in protein which took place during heat treatment. In the presence of nutrient, there was an increase in cell population to six times the original. The increase in population was matched almost precisely by the increases in protein which took place during the two periods.

It should be clear from Fig. 1 that at the end of the period of cell division, both in the presence and in the absence of nutrient, the amount of protein per cell is practically the same as that of the initial log phase cell. Further, it should be evident that the protein content of the cell mass is a good index of cell growth during the period of temperature cycling, and that a single cell count at the end of five and one-half hours, in the absence of nutrient, is a sound measure of the incidence of cell division. These simple tests have been used to evaluate the actions of chloramphenicol and azaserine.

Effect of Chloramphenicol on Cell Growth and Cell Division.—In Fig. 2, the results of the effect of chloramphenicol on cell growth and cell division,

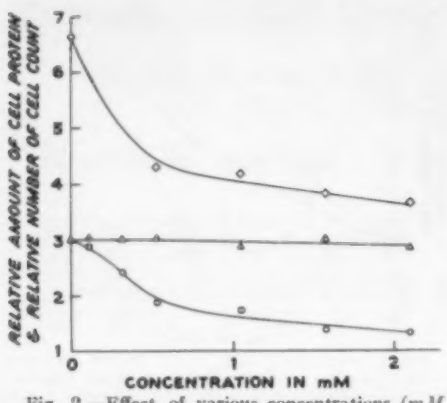

Fig. 2.—Effect of various concentrations (m*M* per liter) of chloramphenicol on cell protein and cell count. ○ = Relative amount of cell protein at the end of heat-treatment period in nutrient medium. ◇ = Relative cell count five and one-half hours after heat treatment in nutrient medium. △ = Relative cell count five and one-half hours after heat treatment in nutrient-free medium.

as measured by protein content and cell count, are expressed in relation to the initial values for each parameter. In the absence of chloramphenicol, the amount of cell protein increased during the period of heat treatment to three times the initial value. Under identical conditions, in the presence of graded concentrations of chloramphenicol, there is a definite inhibition on protein synthesis (Fig. 2). The extent of inhibition is directly related to the concentration of drug present.

In the presence of the same range of concentrations of chloramphenicol as used in the growth studies, cell division in the absence of nutrient is not influenced to any measurable extent (Fig. 2). However, when cell division is permitted to take place in the presence of nutrient, chloramphenicol is definitely inhibitory. Clearly, the inhibitory effect of the drug under these conditions is a consequence of its primary effect on cell growth.

Effect of Azaserine on Cell Growth and Cell Division.—The effect of azaserine on cell growth and cell division is shown in Fig. 3, which is plotted in the manner indicated for Fig. 2. In exact contrast to the action of chloramphenicol, azaserine, within the concentrations studied, has no effect on cell protein synthesis, but has a definite inhibitory action on cell division. At a concentration of 8 μg. per ml. (4×10^{-5} *M*), cell division is almost completely blocked. This same concentration of azaserine shows very little inhibition on cell growth as indicated in Fig. 3.

The addition of azaserine during the period of heat treatment has no observable effect on the subsequent cell division. In one set of experiments 2 μg. of azaserine was added to each ml. (1×10^{-5} *M*) of culture at the beginning of the period of heat treatment. At the end of heat treatment, the cells were washed with buffer and were observed to give the same number of new cells as did the control culture. The result strongly suggests that azaserine does not affect any process in the growth phase that is connected with subsequent cell division.

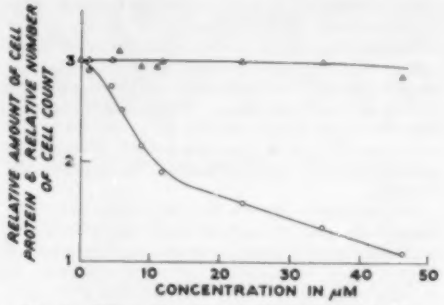

Fig. 3—Effect of various concentrations (μ*M* per liter) of azaserine on cell protein and cell count. △ = Relative amount of cell protein at the end of heat-treatment period in nutrient medium. ○ = Relative cell count five and one-half hours after heat treatment in nutrient-free medium.

DISCUSSION

During the period of temperature cycling, the increase in cell protein observed in this study is almost identical to the increases in dry weight (8), cell volume (9), ribonucleic acid (8), and deoxyribonucleic acid (8) observed by others. This indicates that the growth which takes place during this period, in the absence of drugs, is balanced, in contrast to the unbalanced growth observed by Cohen and Barner (10) in a mutant strain of *E. coli*. The balanced nature of the growth is further supported by the fact that the number of cells that can be derived in nutrient-free medium are directly related to the extent of the increase in growth in the heat-treated cells. In the absence of added drug, any one of the above mentioned parameters may be used to measure cell growth. In the presence of drug, the balance of growth may be disturbed in several possible ways, depending upon the drug and other factors. However, in the present studies, cell protein appears to have served as a valid measure of growth in the presence of either of the two antibiotics.

In the absence of drug, five and one-half hours are required, either in the presence or absence of nutrient, for the completion of the three successive synchronous cell divisions. The difference in cell count in the presence and absence of drug in buffer medium at the end of five and one-half hours serves as a measure of the overall effect of the drug on cell division.

The inhibitory effect of chloramphenicol has been studied in other laboratories (11, 12). The results of the present study indicate that chloramphenicol inhibits the growth of *Tetrahymena pyriformis* under conditions in which cell division does not take place. The results provide experimental evidence that chloramphenicol does not affect cell division at concentrations in which cell growth is inhibited to a great extent. Indeed, it is clear that chloramphenicol inhibits cell division only when that division takes place in nutrient accompanied by cell growth. Clearly, the inhibition of division is a consequence of the inhibition of growth.

The inhibitory action of azaserine on cell regeneration has been reported in a large number of unicellular organisms (13). This is the first time, to our knowledge, its effect has been studied on

Tetrahymena pyriformis. In *Tetrahymena pyriformis*, azaserine definitely inhibits cell division at concentrations which have little or no effect on growth. Its effects on cell division cannot take place during the period of cell growth. Two points which concern the probable mode of action of azaserine are worthy of note. *Tetrahymena pyriformis* requires preformed purines and pyrimidines and cannot synthesize either from precursors (14). In our studies, it has been shown that azaserine inhibits the features of cell division which take place in a nutrient-free medium, after the necessary ribonucleic acid and deoxyribonucleic acid have been accumulated. Accordingly, in these organisms, the drug cannot exert its effect through inhibition of purine synthesis as has been proposed (15).

Studies on the more detailed mechanism of action of these drugs are in progress.

SUMMARY

The controlled-temperature shift technique of Scherbaum and Zeuthen for inducing cell growth and cell division as two distinct processes in mass cultures of *Tetrahymena pyriformis* GL has been restudied. A simplified practical method for measuring cell growth and cell division and the action of drug thereon has been described.

Using this method, the selective activity of two antibiotics, chloramphenicol and azaserine, has been tested. Chloramphenicol, in appropriate concentrations, inhibits protein synthesis, but not cell division; while azaserine, even at a concentration almost completely inhibiting cell division, has very little effect on cell growth.

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Sulfaethylthiadiazole V*

Design and Study of an Oral Sustained Release Dosage Form

By MANFORD J. ROBINSON and JOSEPH V. SWINTOSKY

An aqueous sustained release suspension of sulfaethidole (sulfaethylthiadiazole or SETD) was prepared. The velocity constant (k_b) for drug disappearance from blood in adult human subjects was utilized to approximate the quantity of drug to be administered every twelve hours in order to maintain free SETD blood concentrations near 10 mg. per cent. Dissolution characteristics of this sustained release suspension were also studied *in vitro* in three different test fluids. Possible relationships with the foregoing *in vivo* results are discussed.

THIS PAPER outlines and discusses some of the methods we have utilized in the design, preparation, and evaluation of a liquid sustained release dosage form.

Blythe (1) has discussed general concepts relating to the formulation and evaluation of sustained release products. He describes oral sustained release dosage forms as preparations which provide a sustained therapeutic effect by first releasing a therapeutic dose, then gradually and continually releasing additional medication over a prolonged period. Nelson (2) has discussed the estimation of amounts of drug needed to meet the requirements for sustained release. In previous publications (3–7) we have shown that orally administered sulfaethidole (sulfaethylthiadiazole or SETD) is eliminated from blood at approximately a first-order rate for a considerable period of time following attainment of "pseudo-steady state" diffusion equilibrium. Applications of the use of k_b, the velocity constant for drug elimination from blood, in the design and study of sustained release preparations have been discussed and illustrated (5, 6, 8). Methods for determining rate of dissolution *in vitro* for sustained release products

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have been presented (9–11), although these methods have been designed primarily for control purposes. We have also discussed (8) the possible interrelationships of *in vitro* dissolution rates, blood concentrations, and urinary excretion for two aqueous suspensions of coated sulfamethylthiadiazole.

Recently, an aqueous sustained release preparation of SETD was developed in our laboratories. This drug has been extensively investigated in Europe and found to be a safe, clinically effective sulfonamide (12–15). Recent studies in the United States (16–18) have confirmed the European findings. Frisk (19) has determined blood and urinary concentrations in subjects receiving oral and intravenous doses of SETD. Our investigation of the kinetics of absorption, distribution, and excretion of this drug has been reported (5). Although this drug is normally administered in large doses, the rapidity with which SETD is absorbed and its relatively slower excretion indicated that it could be formulated into a preparation for oral administration every twelve hours. A sustained release form of SETD, by prolonging absorption, should provide relatively uniform, therapeutically effective tissue concentrations for the twelve-hour time period, and also decrease peak loads on the kidney during the early time periods following oral administration. SETD is particularly applicable to this type of study, since it can be readily analyzed in blood and urine and there is a correlation between blood concentration and therapeutic efficacy.

EXPERIMENTAL

Preparation of the Dosage Form.—The sustained release powder was prepared by mixing SETD (40% w/w) with molten hydrogenated castor oil maintained at 110°. The suspension was then spray congealed, using a centrifugal wheel atomizer, to yield a powder having an average surface volume diameter (Fisher Subsieve analysis) of 35 μ. The average surface volume diameter of the SETD used in preparing the sustained release powder was less than 4.0 μ. The sustained release powder was suspended in an aqueous vehicle at a concentration equivalent to 3.9 Gm. of SETD per 30 ml.

In Vitro Dissolution Studies.—The amounts of SETD released in various time intervals for the suspension were determined in three test fluids. Formulas for these fluids and amounts of suspension added in SETD equivalents are shown in Table I. The methods for measuring SETD dissolution in fluids A and B were those previously described for sulfamethylthiadiazole (8).

Test fluid C was developed for control purposes. This fluid was prepared by dissolving the buffer salts in distilled water, adjusting the pH to 6.4 ± 0.03 and, immediately prior to use, adding ox bile extract and pancreatin. Samples of the sustained release suspension equivalent to 0.125 Gm. of SETD were added to 60 ml. of the control fluid in 90-ml. screw-capped containers. These were placed in a constant temperature bath at 30° ± 0.1 and rotated at 40 r. p. m. Samples were removed after one-fourth, one, three, and six hours, filtered, and the residue assayed for SETD content. SETD was extracted from the residue using boiling 1.0 *N* HCl and determined by potentiometric titration with standardized 0.05 *M* $NaNO_2$.

TABLE I.—COMPOSITION OF IN VITRO TEST FLUIDS AND AMOUNTS OF SUSTAINED RELEASE SUSPENSION ADDED IN SETD EQUIVALENT

Test Fluid	pH	Composition	SETD Content
A	1.2	0.1 *N* HCL	2 mg. per cenr
B	8.3	Ox Bile Extract—2.0 Gm., Pancreatin—0.56 Gm. Sodium Bicarbonate—3.0 Gm., Distilled water sufficient to yield 200 ml.	10 mg. per ml.
C	6.4	Citric Acid, hydrous—6.46 Gm., Na_2HPO_4, anhydrous—19.76 Gm., Distilled Water sufficient to yield 1,000 ml. Ox Bile Extract—1.0 Gm., Pancreatin 0.28 Gm. were added to 100 ml. of the buffer solution.	0.125 Gm. per 60 ml.

Blood and Urine Concentration Studies.—The subjects receiving oral doses of the sustained release suspension were healthy, ambulatory adults. The initial dose was administered to each subject immediately before breakfast. Food and fluid ingestion was then permitted *ad libitum*. Blood samples were withdrawn by venipuncture of the antecubital vein. Complete urine collections were made for measurement of total drug output. Dosage regimens and blood and urine sample collection time for these studies were as follows: *Part A.*—12 subjects received single oral doses equivalent to 3.9 Gm. of SETD. Blood samples were withdrawn at two, four, six, eight, ten, and twelve hours. *Part B.*—10 subjects received oral doses equivalent to 3.9 Gm. of SETD initially, followed by 1.95 Gm. every twelve hours for five consecutive doses, totaling approximately 13.65 Gm. Blood samples were taken periodically for seventy-two hours. Complete urine collections were made at twelve-hour intervals for ninety-six hours.

The concentrations of free and free plus conjugated (total) SETD in the blood and urine were determined by previously described procedures (4, 5).

A single lot of the sustained release suspension (X46) was used for the aforementioned studies. To illustrate duration of absorption we have included other unpublished blood concentration and urinary excretion data which were obtained during the development of this product. The data are taken from 144 tests in which single doses of the sustained release suspension were administered orally to 61 adult subjects, and from 5 tests in which 23 adult subjects received single 3.9-Gm. oral doses of crystalline SETD. The procedure of part A was followed except that in the case of the sustained release suspension an additional blood sample was taken at twenty-four hours. Complete urine collections were made

for four, eight, twelve, twenty-four, forty-eight, and seventy-two hours for both the sustained release suspension and crystalline SETD. The lots of the sustained released suspension were of the same composition as lot X46, had approximately the same particle size, and exhibited similar dissolution in the control test fluid (C). The crystalline SETD was administered either in capsules or as an aqueous suspension.

RESULTS AND DISCUSSION

In Vitro Dissolution Studies.—The amounts dissolved and periods for dissolution of the sustained release suspension in test fluids A, B, and C are shown in Table II. Approximately 24% of the SETD was found to dissolve in solution A after fifteen minutes and 41% after one hour. Dissolution in solution B is rapid and can be considered essentially complete (90%) in three hours. Approximately 80% of the SETD is released in test solution C after six hours. Differences in the amount of dissolution in each of the fluids appear to be minimal at the fifteen-minute period. The rate of drug release is not linear and decreases with time after the fifteen-minute period.

TABLE II.—DISSOLUTION OF SETD IN TEST FLUIDS A, B, and C

Time, Hr.	Dissolution, %[a]		
	A	B	C
1/4	24	38	32
1	41	76	60
2	49	..	..
3	..	90	74
6	..	95	81

[a] Average of two or more determinations.

The powder used in preparing lot X46 was examined microscopically in a mineral oil mull and also allowed to disintegrate while being observed under a microscope using chloroform as a solvent for the hydrogenated castor oil. The individual spheres consisted of finely divided SETD particles uniformly dispersed throughout a matrix of hydrogenated castor oil. There was a thin surface film of hydrogenated castor oil distributed uniformly over the surface of each sphere. The observed similarity of initial release and the nonlinear decrease in dissolution rate with time are as would be anticipated for a product having the above physical composition.

Since drug dissolution rate should directly influence absorption, the lack of linearity observed in the *in vitro* tests should be reflected in the observed drug concentration in blood and excretion in urine. As the rate of dissolution in the gastrointestinal tract falls below that necessary to maintain the desired absorption rate a corresponding decrease in blood and urine SETD content would be expected.

Estimate of Drug Dose.—Previous publications (2, 4, 5, 6, 8) have outlined methods for estimating the amount of drug required to maintain a given blood concentration for any time interval. For drugs such as SETD, which are rapidly and completely absorbed and are not significantly degraded or biotransformed, the total sustained release dose may be estimated using the equation, $A = k_b ch + c$, where A equals the total drug dose, k_b is the rate of drug disappearance from blood following attainment of "pseudo-steady state" diffusion equilibrium and essentially complete absorption, c is the amount of drug required to obtain the desired blood concentration, and h is the number of hours that the selected blood concentration is to be maintained following attainment of "pseudo-steady state" diffusion equilibrium and essentially complete absorption. When estimating initial and subsequent sustained release doses for multiple dosage regimens the initial dose is approximately equal to A and subsequent doses A' are approximately equal to $k_b ch$.

We have shown (3–6) that a 2.0-Gm. dose of SETD provides a blood concentration near 10 mg. %, that "psuedo-steady state" diffusion equilibrium is attained, and absorption is essentially complete after about two hours, and that the k_b for SETD disappearance[1] from blood is in the order of .07 to .12. We have shown also (6) that an initial dose of 2.0 Gm. of SETD followed by 0.22 Gm. every hour will provide and maintain free blood concentrations near 10 mg. %.

Estimation of SETD dosage is complicated by a number of factors which are not readily amenable to calculation. It is desirable to limit the dosage form to a single package or unit. Since subsequent as well as initial doses of the sustained release suspension will contain nearly 50% of SETD available for rapid absorption, the blood levels must be allowed to subside somewhat in the later hours prior to administration of subsequent doses. It has been proposed that this occur at ten hours. However, for products which exhibit nonlinear type dissolution and for drugs which are rapidly absorbed and relatively slowly excreted (5) the blood concentrations possibly should subside at an earlier time. Further, the initial and subsequent twelve-hour doses must be such that they can be prescribed in units which are readily measured by the consumer. The consumer may be either an adult receiving a fixed amount of drug or an infant or child receiving the product on a mg./Kg. or gr./lb. basis.

An SETD dosage regimen of 3.9 Gm. for the initial twelve-hour period followed by one-half of the original dose (1.95 Gm.) every twelve hours is compatible with the above factors and is in reasonable accord with the calculated estimate. This dosage regimen should provide blood concentrations near the desired 10 mg. % for the twelve-hour time interval. The 3.9-Gm. SETD dose corresponds to an initial dose near 2 Gm. to hasten the approach to the steady state and approximately 1.9 Gm. to maintain this blood concentration.

Blood and Urine Concentration Studies.—*Part A.*—The individual blood concentrations observed for 12 subjects receiving a single dose of the sustained release suspension (X46) equivalent to 3.9 Gm. of SETD are shown in Table III. The minimum, maximum, and average free SETD blood concentrations for these subjects are shown in Fig. 1. The mean blood concentrations are near 10 mg. % for the observed ten hours and are in reasonable correspondence with those found for similar subjects receiving an initial 2.0-Gm. dose of SETD followed by 0.22-Gm. doses of SETD every hour (6). This is further illustrated in Table IV for the only subject (Ja) receiving both dosage regimens. The correspondence in blood concentrations indicates that the

[1] k_b here is .07 to .12 hour^{-1}, or may be expressed as 7 to 12% per hour.

sustained release suspension is providing an initial release near 50% of SETD and that the balance is being absorbed over most of the observed time period.

The average total excretion in urine four hours after oral administration of crystalline SETD in 1.0, 2.0, and 3.0-Gm. doses previously observed for 4 subjects was 200, 460, and 775 mg., respectively. The average total excretion in urine for subjects receiving single doses of the sustained release suspension (X46) was 466 mg. after four hours. The close correspondence in the four-hour excretion values for a 2.0-Gm. dose of crystalline SETD and a single dose of the sustained release suspension also indicates that approximately 50% of the drug content is available for early absorption from the suspension.

Average free blood concentrations over a twenty-four hour period for 61 subjects receiving a single dose of the sustained release product equivalent to 3.9 Gm. of SETD are shown in Fig. 2. Cumulative drug excretion in urine for 49 of these subjects over seventy-two hours is shown in Table V. These data are in reasonable agreement with those observed for lot X46. It has been reported previously (4, 8) that absorption may be considered essentially terminated when the slope of the first-order rate equation plots of drug disappearance from blood becomes identical for the dosage forms studied. For this product the semilogarithmic plot of the average free SETD blood concentration approximates a straight line after six hours. The slope of this line, however, indicates a rate of disappearance (k_b) near .05 hour^{-1} rather than .07 to .12 hour^{-1} observed for crystalline SETD. This difference in SETD excretion is also illustrated in Table V which compares the average cumulative drug excretion in urine over a seventy-two-hour period for 23 adult subjects receiving crystalline SETD with that for 49 adult subjects receiving the sustained release product. These data indicate that absorption is apparently occurring after six hours, but at a rate which with time becomes increasingly less than that required to maintain a constant blood concentration.

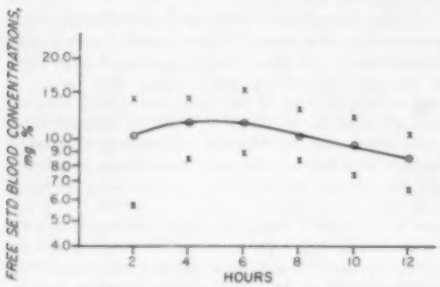

Fig. 1.—Minimum, maximum, and average free SETD blood concentrations for 12 adult human subjects receiving a single oral dose of the sustained release suspension X46 equivalent to 3.9 Gm. of SETD.

The indication that approximately 2.0 Gm. is available for early absorption and the prolonged release which may be inferred from the extended absorption time agree generally, but do not correlate precisely, with the results which were anticipated from the *in vitro* dissolution study.

Part B.—Clinical studies have shown the therapeutic efficacy and safety of SETD when administered according to a dosage regimen of 1.0 Gm. every six hours (16). It has been shown (6) that in the type of subject being studied such a regimen will provide steady state total SETD blood concentrations lying between 6.5 and 18 mg. %. The sustained release product should provide comparable

TABLE III.—FREE BLOOD CONCENTRATIONS (mg. %) FOR ADULT HUMAN SUBJECTS RECEIVING A SINGLE ORAL DOSE OF SUSTAINED RELEASE SUSPENSION (X46) EQUIVALENT TO 3.9 GM. OF SETD

Subject	Hours 2	4	6	8	10	12
Br	14.3	14.2	12.8	10.9	9.6	8.5
Ca	11.6	14.0	15.4	13.0	12.1	10.5
Cr	11.6	10.3	8.9	8.4	7.9	7.5
Ha	11.3	12.2	9.8	8.4	7.4	7.2
Jo	5.7	8.5	10.5	9.0	8.8	8.4
Bo	6.7	10.1	10.8	9.5	9.4	9.0
Ra	9.8	10.1	10.8	11.0	10.3	9.4
Ch	12.5	12.3	12.0	10.5	10.3	8.4
Mi	10.8	11.4	11.6	10.3	9.8	8.8
Jn	10.5	14.0	13.0	11.8	10.3	9.4
Cl	9.8	10.5	10.8	9.8	9.4	8.5
Je	10.1	10.3	11.4	10.8	8.1	6.5

TABLE V.—AVERAGE SETD EXCRETION IN URINE OVER A SEVENTY-TWO-HOUR PERIOD FOR 49 ADULT SUBJECTS RECEIVING A SINGLE ORAL DOSE OF THE SUSTAINED RELEASE PRODUCT EQUIVALENT TO 3.9 GM. OF SETD AND 23 ADULT SUBJECTS RECEIVING ORAL DOSES OF APPROXIMATELY 3.9 GM. OF CRYSTALLINE SETD

Collection Time	Average SETD Excretion in Urine, mg. SETD	Sustained Release Product
0–4	1149	451
4–8	821	720
8–12	407	473
12–24	608	714
24–48	472	648
48–72	106	202

TABLE IV.—FREE AND TOTAL BLOOD CONCENTRATIONS FOR SUBJECT JA FOLLOWING A SINGLE ORAL DOSE OF THE SUSTAINED RELEASE SUSPENSION (X46) EQUIVALENT TO 3.9 GM. OF SETD AND ORAL DOSES OF 2.0 GM. FOLLOWED BY 0.22 GM. EVERY HOUR FOR EIGHT HOURS OF SETD

Material	Hours 1	2	4	6	8	10	12
SETD capsules							
Total	9.9	13.2	13.4	12.3	11.2	..	9.3
Free	9.5	12.8	12.3	11.8	11.0	..	8.7
Sustained release (X46) suspension							
Total	..	9.0	11.5	12.7	11.1	10.2	9.3
Free	..	8.8	10.9	11.5	9.7	9.4	8.6

blood concentrations when a dose approximating 2.0 Gm. of SETD is administered every twelve hours. The observed total SETD blood concentrations for 10 subjects receiving oral doses of the sustained release suspension (X46) equivalent to 3.9 Gm. of SETD initially followed by 1.95 Gm. of SETD every twelve hours for 5 consecutive doses are shown in Fig. 3 (the free drug concentrations were only slightly lower, as has been noted previously (6)). Circles denoting the average concentration in blood have generally been connected by lines. These lines do not necessarily indicate the precise concentration at any time but were added to aid in visualization. The vertical distances between the starred points denote the limits of observed total drug concentration in blood. The mean experimental values shown in Fig. 3 are reasonably constant throughout the entire period, and the concentrations generally are within the theoretical limits calculated for a dosage regimen of 1.0 Gm. of crystalline SETD administered every six hours. The blood concentrations also correspond closely to those previously reported (6) for similar subjects receiving 2.0 Gm. of crystalline SETD followed by 1.0-Gm. dose every six hours for 11 consecutive doses. For purposes of comparison these data are shown in Fig. 4

Figure 5 illustrates the constant twelve-hour excretion pattern of SETD following establishment of diffusion equilibrium and steady state blood concentration. Absorption efficiency may be calculated from the slope of the line during its straight line phase. The theoretical slope for this dosage regimen, when absorption and urinary excretion efficiency are 100%, is 0.163 Gm./hour The slope calculated from Fig. 5 is 0.16 Gm./hour, indicating an average absorption efficiency greater than 95% for these subjects. Total SETD recoveries in urine after ninety-six hours for the subjects receiving the dosage regimens shown in Figs. 3 and 5 ranged from 87–100% with an average of approximately 93%. An average of 6.5% of the drug found in urine was biotransformed. This amount of biotransformation, based on urinary data, compares favorably with the 11% previously reported (6) for similar subjects receiving multiple oral doses of crystalline SETD.

These data for multiple doses of the sustained release suspension indicate that when SETD is incorporated in sustained release form for twice daily oral dosage the physician may expect to obtain blood concentrations at least equivalent to those previously obtained on a four times daily regimen. Further, the prolongation of absorption time through sustained release does not decrease the efficiency of total absorption nor increase the per cent biotransformation of this drug.

These experimental data in conjunction with previously reported data (3, 4, 5, 6, 8) illustrate some of

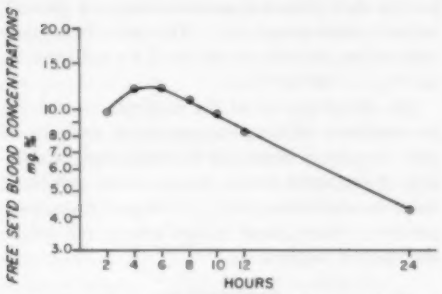

Fig. 2.—Average free SETD blood concentrations for 61 adult human subjects receiving a single oral dose of the sustained release product equivalent to 3.9 Gm. of SETD.

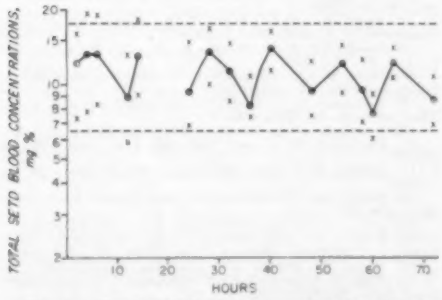

Fig. 3.—Total SETD blood concentration data for 10 adult human subjects receiving the sustained release suspension X46 in doses equivalent to 1.95 Gm. every twelve hours. Broken lines indicate calculated maximum and minimum limits.

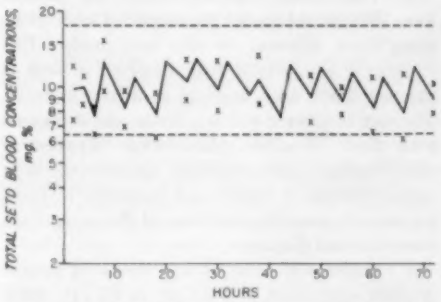

Fig. 4.—Total SETD blood concentration data for 4 adult human subjects (6) receiving 2 Gm. SETD followed every six hours by a 1-Gm. dose for seventy-two hours. Broken lines indicate minimum and maximum calculated limits where the k_b is estimated to vary between .07–.12 hour^{-1} and the time lag for absorption T_l is estimated to be 2.0 hours.

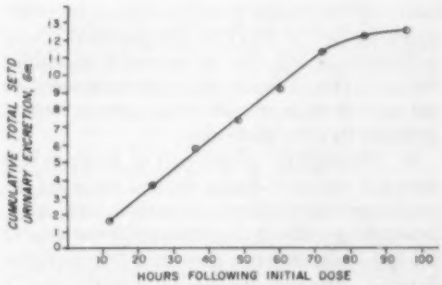

Fig. 5.—Average cumulative urinary excretion of total SETD for 10 adult subjects corresponding to the dosage regimen shown in Fig. 3.

the methods we have utilized for the design and evaluation of a sustained release dosage form. The utility of the specific velocity constant for drug elimination from blood for estimating amounts of drug to be incorporated into and approximating the amount and duration of *in vivo* release for sustained release dosage forms is discussed. The general relationships between results anticipated by *in vitro* tests and those observed *in vivo*, although not precise, may aid in the formulation of oral sustained release dosage forms.

This approach should not only facilitate sustained release dosage form study and design, but may also aid in establishing standards of performance for some sustained release products.

SUMMARY

1. A sustained release suspension of sulfaethylthiadiazole (SETD) was prepared by spray congealing a mixture of SETD (40 per cent w/w) in molten hydrogenated castor oil, and suspending the resulting powder in an aqueous vehicle.

2. The spray-congealed powder consisted of spherical particles having an average particle size of approximately 35 μ. Fine particles of SETD were uniformly distributed within the spheres.

3. Dissolution characteristics of the powder were determined under standardized conditions using three different *in vitro* test fluids. Differences in amounts of drug dissolved in each of the test fluids were minimal at fifteen minutes. The rate of release was not linear and decreased with time. Possible relationships between *in vitro* dissolution characteristics, the observed drug concentrations in blood, and excretion in urine for subjects receiving oral doses of the product are presented and discussed.

4. Single oral doses of the sustained release product equivalent to 3.9 Gm. of SETD, when administered to 12 adult human subjects, maintained blood concentrations near 10 mg. per cent during a two to twelve-hour period. Maximum concentrations occurred near six hours and subsided to the minimum at twelve hours.

5. Blood concentrations after two hours and the amount of drug collected in urine after four hours are very similar for subjects receiving orally either 3.9 Gm. of SETD in the sustained release suspension or 2.0 Gm. of uncoated crystalline SETD. This indicates that approximately 50 per cent of the sustained release dosage form is available for early absorption.

6. Although the greater part of the single oral dose was absorbed during the first six hours the remainder was apparently absorbed over an extended time interval, as evidenced by the slope of the blood concentration curve and drug excretion in urine. The apparent mean k_b from the slope of the straight line after six hours for the subjects studied was approximately .05 hour^{-1}, whereas for similar subjects receiving nonsustained crystalline SETD the actual k_b value ranged from .07 to .12 hour^{-1}. The amount of SETD excreted in urine over seventy-two hours for subjects receiving the sustained release product was found to be less in early and more in later time periods than for similar subjects receiving crystalline SETD.

7. Administration of multiple oral doses of the sustained release suspension equivalent to 3.9 Gm. of SETD for the first twelve hours followed by 1.95 Gm. every twelve hours resulted in blood concentration maxima and minima which correspond to those calculated and observed for a regimen of 2.0 Gm. of uncoated crystalline SETD for the first six hours followed by 1.0 Gm. every six hours.

8. Drug recovery from urine was used to determine minimum absorption efficiency under the conditions of this study. About 95 per cent of the administered dose was recovered in urine, an amount corresponding to that observed when crystalline SETD is administered.

9. Biotransformed SETD in urine averaged 6.5 per cent following administration of the sustained release suspension. This was of the same order of magnitude as observed for subjects receiving crystalline SETD.

10. Employment of the concepts embodying measurement of drug disappearance from blood and excretion in urine may facilitate study and design of sustained release dosage forms and be of value in establishing standards of performance for products where these values can be related to therapeutic response.

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Spectrophotofluorometry of Reserpine, Other Rauwolfia Alkaloids, and Related Compounds*

By R. P. HAYCOCK, P. B. SHETH, and W. J. MADER

The excitation and fluorescent characteristics of a number of Rauwolfia alkaloids and related compounds are reported using a Spectrophotofluorometer. A procedure for the determination of reserpine in mixtures of reserpine and rescinnamine is described.

THE PHENOMENA of natural fluorescence of reserpine and other rauwolfia alkaloids has been noted by various investigators since the isolation of reserpine was reported, and several investigators have utilized fluorescences for the determination of reserpine in dosage form and in biological tissues (1–3). However, these investigators employed filter type fluorometers which restrict the fluorescent detection to the visible region of the spectrum and to those fluorophors that absorb light corresponding to one of the available lines of the mercury emission spectrum. The development of spectrophotofluorometers has extended the scope of fluorescent analysis to permit the excitation of compounds and the measurement of resulting fluorescence throughout the ultraviolet and visible regions.

To date, little data have been reported on scanning spectrophotofluorometric measurements of rauwolfia alkaloids. This led to a survey of the excitation and fluorescent characteristics of a number of rauwolfia alkaloids and related compounds for possible usefulness as a basis for the identification and quantitative assay of reserpine in the presences of other rauwolfia alkaloids.

EXPERIMENTAL

Apparatus.—Aminco-Bowman Spectrophotofluorometer with silica cells, 1-cm. cross section, four transparent sides. Recorded spectra were obtained, using a Mosely Model No. 3 Flat Bed X-Y Recorder, modified for use with the Spectrophotofluorometer.

Solutions.—Dissolve 25.0 mg. of the compound in 1 ml. of chloroform and dilute to 100 ml. with methanol. Dilute 3.0 ml. to exactly 50 ml. with methanol and further dilute 10 ml. to exactly 50 ml. with methanol. The diluted solution contains 3.0 μg. of alkaloid per ml. Indole, norharmane, and harmaline are prepared to contain 1.5 μg. per ml.

Technique.—The Aminco-Bowman Spectrophotofluorometer used in this study was calibrated using 0.1 μg. U. S. P. quinine sulfate per ml. in 0.1 *N* sulfuric acid in accordance with the technique of Sprince and Rowley (4). The excitation maximum was observed at 350 mμ, and the corresponding maximum fluorescent peak occurred at 450 mμ. The peaks checked within 5 mμ on repeated tests and agreed with the corrected values reported by Sprince and associate.

The fluorescent spectrum for each solution was scanned on the oscilloscope screen, while changing the excitation wavelength until the appearance of a fluorescent band. At the fluorescent maximum, the wavelength of maximum excitation was determined by manipulation of the excitation monochromator. Under these conditions, excitation and fluorescent maxima were obtained for each compound tested, as shown in Table I, together with the structural formula and relative fluorescent sensitivity. The relative fluorescent sensitivity is the product of the meter reading on the transmission scale and meter multiplication factor. Excitation and fluorescent spectra of reserpine, rescinnamine, 3-dehydroreserpine, and tetrahydroreserpine are presented in Figs. 1–4. These spectra are typical of the other alkaloids and related compounds.

METHOD DEVELOPMENT

An adaptation of the Szalkowski-Mader colorimetric reaction of reserpine with nitrous acid is the official (5) and preferred procedure for determining reserpine in dosage form. In a prior publication (6) from this laboratory, it was established that the 7-methoxy-β-carboline group, i.e., the 11-methoxy group and the AB and C ring skeleton of reserpine, is the functional group responsible for the yellow pigment which is formed. Of the alkaloids containing this similarity to reserpine, methyl reserpate and reserpic acid are eliminated in the extraction procedure and dehydroreserpine and tetradehydroreserpine do not interfere. The other principal alkaloid found in various species of rauwolfia which contains this common structural similarity to reserpine and reacts with nitrous acid is rescinnamine. A preliminary examination of the fluorescent spectra of reserpine and rescinnamine showed that they exhibit different excitation and fluorescent spectra and led to a study of the practicability of determining reserpine in the presence of rescinnamine by spectrophotofluorometry.

Procedure.—A solution of reserpine in methanol was prepared as outlined under Experimental to give a range of 1 μg. to 5 μg. per ml. The excitation monochromator and fluorescent monochromator were set at 280 mμ and 360 mμ, respectively, at the maximal excitation and fluorescent response of reserpine. The relationship between concentration of reserpine and fluorescence is shown in Fig. 5. All measurements were made using $^1/_{16}$ inch defining slit (band pass = 6 mμ) and a 1P21 photomultiplier. The blank reading served as the fiducial point, and the instrument was set at an arbitrary point with the highest standard. The fluorescence is proportional to concentration in the range of 1 to 5 μg. per ml.

The above method has been successfully applied to mixtures of reserpine containing as much as 80%

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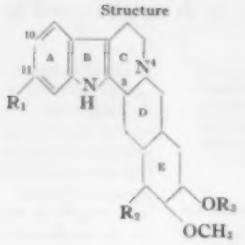
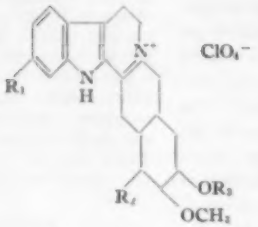
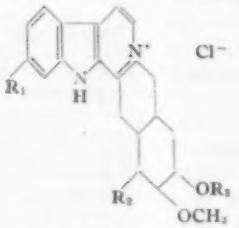
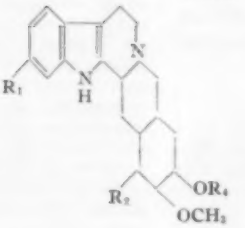
TABLE I.—SENSITIVITY AND MAXIMUM EXCITATION AND FLUORESCENT WAVELENGTHS FOR SOME RAUWOLFIA ALKALOIDS AND RELATED INDOLES[a]

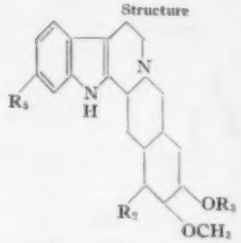
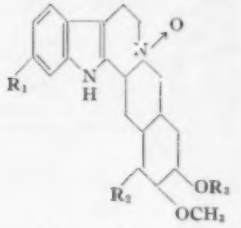
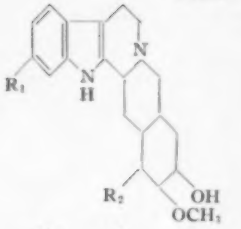
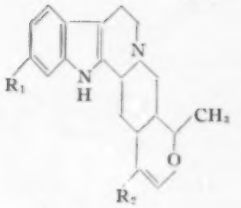
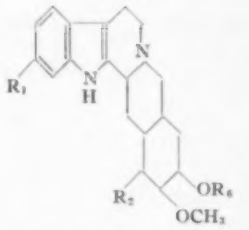
Compound	Structure	Wavelength, mμ Excitation, max.	Wavelength, mμ Fluorescence, max.	Relative Fluorescent Sensitivity
Reserpine		280	360	6
Isoreserpine	.....	390	510	18
Dehydroreserpine perchlorate		390	510	75
Tetradehydroreserpine chloride		340	440	50
Rescinnamine		310	440	2

rescinnamine with recoveries within 2 to 3% of the theoretical reserpine content as shown in Table II. The reproducibility of replicate determinations was within 3%. This difference is not more than can be accounted for by analytical errors.

DISCUSSION

The following discussion is not meant to be a complete evaluation of the findings, rather it is intended only to point out the more evident results to emerge from the investigation. The excitation and fluorescent spectra of reserpine, reserpinine, deserpidine, renoxidine (reserpine N-oxide), methyl reserpate, methyl O-(3,5-dimethoxy-4-hydroxybenzoyl) reserpate, syrosingopine, and syrosingopine N-oxide generally showed peaks at approximately 280 mμ with a corresponding fluorescent maximum at approximately 360 mμ. The order of magnitude of fluorescence depends upon certain groups in various positions of the basic alkaloid structure. For example, the 11-methoxy moiety impedes fluorescences as is evident by a comparison of reserpine with deserpidine, which does not possess an 11-methoxy group and produces an increase in fluorescences equivalent to $2^1/_2$ above that of reserpine. Reserpinine, a tertiary indole alkaloid with a heterocyclic ring E and possessing an 11-methoxy substituent emits fluorescent light below that of reserpine. The introduction of oxygen at position 4, however, is an enhancing group as is evident by an increase in the fluoresence emission

TABLE I—*Continued*

Compound	Structure	Wavelength, mμ Excitation, max.	Wavelength, mμ Fluorescence, max.	Relative Fluorescent Sensitivity
Deserpidine		280	360	15
Renoxidine (reserpine N-oxide)		280	360	8
Methyl reserpate		300	360	19
Reserpinine		300	360	2
Methyl O-(3,5-dimethoxy-4 hydroxybenzoyl) reserpate		300	360	8

of renoxidine (reserpine N-oxide) and syrosingopine N-oxide. The esterified alcoholic function of reserpine, i. e., trimethoxybenzoic acid decreases the fluorescences considerably as can be seen by a comparison of the fluorescent emission of reserpine with methyl reserpate. The substitution of a hydroxyl group in the esterified function, i.e., 3,5-dimethoxy-4-hydroxybenzoic acid has an enhancing effect upon the fluorescence displayed by reserpine as is evident by the increased fluorescences of methyl O-(-3,5-dimethoxy-4-hydroxybenzoyl) reserpate.

The esterified alcoholic function of rescinnamine, i. e., trimethoxycinnamic acid, is responsible for a shift in the excitation maximum to 310 mμ and a corresponding shift in the fluorescent emission to 440 mμ. In addition to the shift, the fluorescent response is considerably weaker than that produced by the reserpine nucleus. This qualitative and quanti-

TABLE I—*Continued*

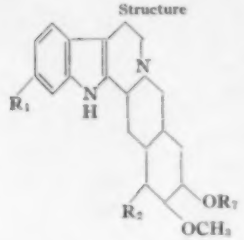
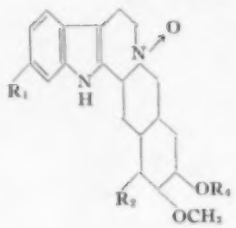
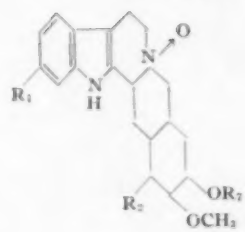
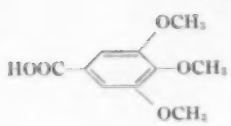
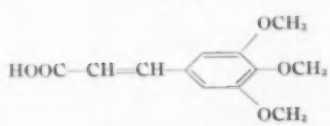
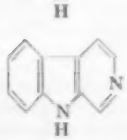
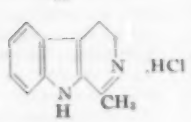
Compound	Structure	Wavelength, mμ Excitation, max.	Wavelength, mμ Fluorescence, max.	Relative Fluorescent Sensitivity
Syrosingopine		300	360	4
Rescinnamine N-oxide		310	440	2–3
Syrosingopine N-oxide		290	350	6
Trimethoxybenzoic acid		280	360	2
Trimethoxycinnamic acid		300	400	<1
Indole		280	340	36
Norharmane		350 285	380 380	36 40
Harmaline hydrochloride		390	490	150

TABLE I—*Concluded*

Compound	Structure	Wavelength, mμ Excitation, max.	Wavelength, mμ Fluorescence, max.	Relative Fluorescent Sensitivity
Key:				
	$R_1 = CH_3O$			
	$R_2 = COOCH_3$			
	$R_3 = OC$–(3,4,5-trimethoxybenzene)			
	$R_4 = OC{-}CH{=}CH$–(3,4,5-trimethoxybenzene)			
	$R_5 = H$			
	$R_6 = OC$–(3,5-dimethoxy-4-hydroxybenzene)			
	$R_7 = OC$–(3,5-dimethoxy-4-$OCOOC_2H_5$benzene)			

[a] The authors wish to acknowledge the assistance of their associates, R. A. Lucas and P. R. Ulshafer, who supplied us with some of the alkaloids.

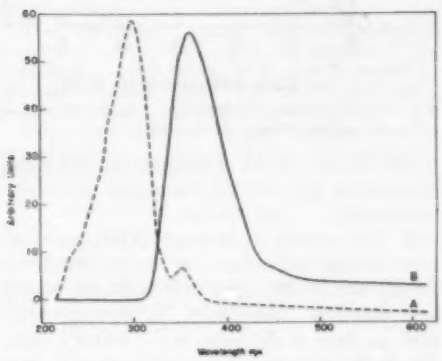


Fig. 1.—Excitation and fluorescent spectra of reserpine in methanol (3 μg./ml.). A. Excitation scan—fluorescence held at 360 mμ. B. Fluorescent scan—excitation held at 280 mμ.

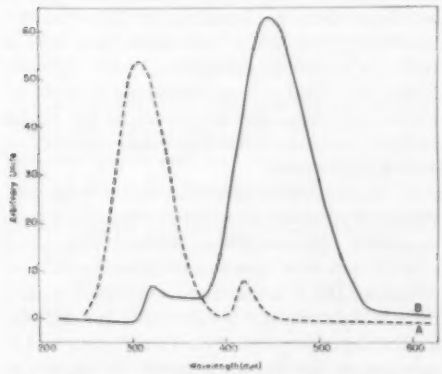


Fig. 2.—Excitation and fluorescent spectra of rescinnamine in methanol (3 μg./ml.). A. Excitation scan—fluorescence held at 440 mμ. B. Fluorescent scan—excitation held at 315 mμ.

TABLE II.—ANALYSES OF RESERPINE-RESCINNAMINE MIXTURES

Reserpine Added, %	Rescinnamine Added, %	Reserpine Found, %
99.2	0.8	98.5
99.2	0.8	99.2
95.2	4.8	95.2
95.2	4.8	94.2
80.0	20.0	80.0
80.0	20.0	81.5
40.0	60.0	40.9
25.0	75.0	25.8
16.7	83.3	17.2

tative difference can be utilized in the determination of reserpine in the presence of rescinnamine. However, it is not applicable when applied to preparations containing other rauwolfia alkaloids having identical fluorescent characteristics to that of reserpine unless used in conjunction with other techniques. For example, methyl reserpate and reserpic acid may be removed by preliminary extraction with acid and base as outlined in the U. S. P. nitrite procedure (5). The presence of deserpidine, a possible contaminant of commercial reserpine obtained from *Rauwolfia canescens*, may be detected by chromatography (7) on paper and on columns or may be determined by difference between the spectrophotofluorometric technique and the U. S. P. nitrite procedure (5), since deserpidine does not react with nitrous acid. In spite of its limitations, the spectrophotofluorometry technique embodies certain advantages not inherent in other procedures and, in particular, may be applied to the determination of micro quantities of reserpine.

Dehydroreserpine and 3-isoreserpine display a high visible fluorescence at 510 mμ, which is maximally excited at 390 mμ. This shift in fluorescences of isoreserpine from the ultraviolet is not accompanied by a noticeable change in the absorption spectrum of the basic reserpine nucleus. The fluorescence of dehydroreserpine is equivalent to twelvefold that of reserpine, and the fluorescent emission of iso-

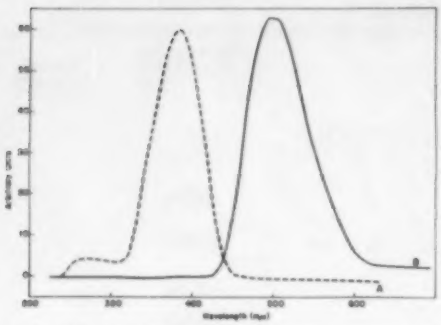


Fig. 3.—Excitation and fluorescent spectra of 3-dehydroreserpine in methanol (3 μg./ml.). A. Excitation scan—fluorescence held at 510 mμ. B. Fluorescent scan—excitation held at 390 mμ.

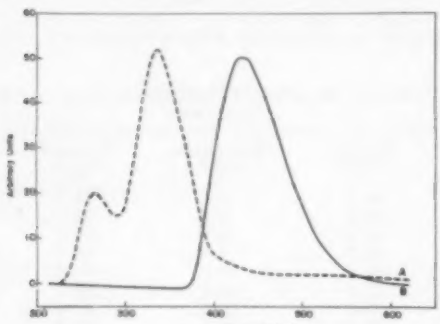


Fig. 4.—Excitation and fluorescent spectra of tetradehydroreserpine in methanol (3 μg./ml.). A. Excitation scan—fluorescence held at 440 mμ. B. Fluorescent scan—excitation held at 340 mμ.

reserpine is increased by a factor of three over that of reserpine. Presumably, this shift in fluorescences is related to epimerization at C-3 and unsaturation in ring C. Although the significance of this phenomenon is not understood, it can be useful as a method for assaying reserpine, as the visible fluorescence of dehydroreserpine is increased 12 times that of the natural fluorescences of reserpine and offers the added advantage of the possibility of using filter type fluorometers. Details of a procedure based on the conversion of reserpine to dehydroreserpine to produce a fluorescent peak at 510 mμ will be the basis of a subsequent publication.

An examination of tetradehydroreserpine, another oxidative degradation product of reserpine, likewise displays a sizeable increase of fluorescence over that of reserpine. In addition to the order of magnitude of fluorescence, the excitation and fluorescence emission are unlike those of either reserpine or dehydroreserpine. In this case the maximal fluorescent emission is found at 440 mμ when excited at 340 mμ.

CONCLUSIONS

1. A large number of rauwolfia alkaloids and related compounds have been subject to a spectrophotofluorometric study, and for each compound the wavelength of maximal excitation and fluorescence and relative fluorescent sensitivity are reported.

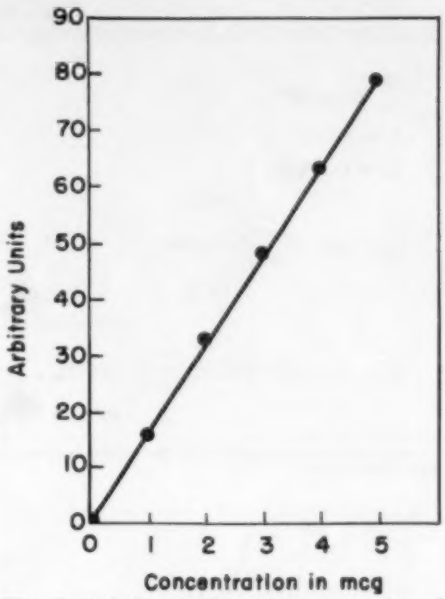


Fig. 5.—Relationship between concentration of reserpine and fluorescence.

2. The totality of the results of this survey allows several generalizations as to structural groups and fluorescence. The methoxy moiety and esterified substituent, trimethoxybenzoic acid, produces a decrease in the emitted light. The degree of fluorescence of indole alkaloids with a heterocyclic ring E is less than that of the reserpine nucleus. The introduction of oxygen at position 4 enhances the fluorescent emission. The substitution of trimethoxycinnamic acid as the esterified alcoholic function in place of trimethoxybenzoic acid is responsible for a shift as well as a weaker fluorescent response. Epimerization at C-3 and unsaturation in ring C shifts the fluorescence from the ultraviolet to the visible with an increase in the fluorescent intensity of several magnitudes.

3. A fluorometric procedure based on the conversion of reserpine to dehydroreserpine has been suggested. Details will be published later.

4. A practical spectrophotofluorometric procedure for the determination of reserpine in mixtures of reserpine and rescinnamine has been described that is based on the natural fluorescence of reserpine at 360 mμ when maximally excited at 280 mμ. Rescinnamine exhibits different excitation and fluorescent spectra and does not interfere.

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A Note on N-Ethoxy-N-ethyl-*m*-toluamide Insecticide*

By RANDOLPH T. MAJOR and HANS-JURGEN HESS†

Based on structural relationships it was postulated that N-ethoxy-N-ethyl-*m*-toluamide would prove to be an effective insect repellent as is N,N-diethyl-*m*-toluamide. It has been shown that N-ethoxy-N-ethyl-*m*-toluamide is an insecticide, particularly against mosquitoes.

RECENTLY, investigators in the U. S. Department of Agriculture have reported that N,N-diethyl-*m*-toluamide is an active insect repellent (1–3).

Based on analogies between the pharmacological activity of substituted amines and substituted hydroxylamines (4) it seemed likely that N-ethoxy-N-ethyl-*m*-toluamide, *m*-$CH_3C_6H_4CON(C_2H_5)OC_2H_5$, would also be an insecticide or insect repellent.

N-Ethoxy-N-ethyl-*m*-toluamide was prepared by the interaction of *m*-toluyl chloride (5) and N-ethoxy-ethylamine (6, 7).

EXPERIMENTAL

N-Ethoxy-N-ethyl-*m*-toluamide.—To 10.6 Gm. (0.069 mole) of *m*-toluyl chloride in 10 cc. of benzene was added, with external cooling, 13 Gm. (0.146 mole) of N-ethoxy-ethylamine[1] in 10 cc. of benzene. After the mixture had stood, overnight, it was treated with an aqueous solution of potassium carbonate and extracted with ether. The ether solution was dried over sodium sulfate and distilled, b.p. of the amide, 137–142° at 4.5 mm. Redistilled, b. p. 139° at 4.5 mm., yield, 10.6 Gm. (75%).

Anal.—Calcd. for $C_{12}H_{17}NO_2$: C, 69.51; H, 8.27; N, 6.76. Found: C, 69.60; H, 8.57; N, 7.05.

Biological Activity.—Dr. R. F. Phillips of the Merck Sharp and Dohme Research Laboratories has reported that the compound showed no antibacterial or antifungal activity against common plant pathogens. However, he has written us that Dr. Philip Granett of the Department of Entomology of Rutgers University put some mosquitoes, *Aedes aegypti*, in a five-inch long by two-inch diameter tube and tied a piece of cheesecloth which had been treated with a 5% solution of N-ethoxy-N-ethyl-*m*-toluamide in acetone on one end of the tube. The mosquitoes were knocked down, i. e., paralyzed or killed rapidly. Also, a moderate degree of repellency was noted against stable flies, *Stomoxys calcitrans*, but not against house flies, *Musca domestica*. Dr. Phillips has also written us that Dr. H. M. Peck of the Merck Institute for Therapeutic Research has carried out preliminary studies of the toxicity of N-ethoxy-N-ethyl-*m*-toluamide. These indicate that it is probably relatively nontoxic. No indication of dermal injury to the skin of the rabbit was noted when the material was tested for twenty-four hours by the Draize patch test. Toxicity tests in Carsworth CF strain mice gave the following results:

i.p. LD_{50}—approx. 800 mg./Kg.
P.O. LD_{50}—approx. 2.0 Gm./Kg.

Toxic signs at such levels were loss of righting reflex, sedation, exophthalmia, ataxia, lacrimation, and slow respiration.

SUMMARY

1. N-Ethoxy-N-ethyl-*m*-toluamide has been synthesized.

2. Preliminary toxicity tests indicate that N-ethoxy-N-ethyl-*m*-toluamide is relatively nontoxic systemically, and also to the skin.

3. N-Ethoxy-N-ethyl-*m*-toluamide shows considerable toxicity to mosquitoes, *Aedes aegypti*, and repells stable flies, *Stomoxys calcitrans*, but not house flies, *Musca domestica*.

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* Received March 27, 1959, from the Cobb Chemical Laboratory, University of Virginia, Charlottesville.

The authors are indebted to Merck and Co., Inc., for a grant in support of this research and to the several investigators mentioned in this paper who have cooperated with them.

† Merck and Co., Inc., Post-Doctoral Fellow in Chemistry, 1957–1958.

[1] It should be noted that on one occasion a violent explosion occurred during the preparation of N-ethoxy-ethylammonium chloride by the method of R. T. Major and E. E. Fleck, *J. Am. Chem. Soc.*, **50**, 1479(1928). An aqueous solution of this salt was being concentrated *in vacuo* in an electric heating mantle. Apparently, the salt was overheated. Aqueous solutions of N-ethoxy-ethylammonium chloride should be concentrated to dryness on a water bath or a steam bath, preferably *in vacuo*.

Sensitivity to Vaginal Jellies: A Note on the Therapeutic Index of Spermicides*

By JOHN H. HOLZAEPFEL, JOHN S. WARNER, and JULIET A. HOWARD

IN A RECENT PUBLICATION (1) we described the development of a rabbit vaginal-irritation test that is recommended as a reliable procedure for the determination of irritating properties of vaginal jellies and creams. The present communication describes some of the results that we have obtained in using that procedure to evaluate the irritating properties and to determine the therapeutic indexes of experimental spermicides in jelly formulations.

Two main criteria of a satisfactory contraceptive jelly are effectiveness and safety. In an investigation to develop an improved contraceptive formulation, an extensive study (2) of the spermicidal effectiveness of chemical compounds was made by using a modification of the Brown and Gamble (3) screening procedure. A number of effective spermicides that had not been previously reported were discovered.

It was then necessary to determine the safety of these compounds in contraceptive formulations. In many cases the available toxicity data indicated that the materials were harmless. The rabbit vaginal-irritation test was used to determine another aspect of safety; namely, irritating properties. In contraceptive formulations as in most pharmaceutical products, the therapeutic index of an active ingredient must be considered as important as effectiveness alone. In our work, the therapeutic index was considered to be the ratio of the maximum nonirritating concentration in the rabbit vaginal-irritation test to the minimum concentration showing instantaneous immobilization of sperm in the modified Brown and Gamble test. Table I shows the results from the evaluation of a number of

TABLE I.—DATA FROM RABBIT VAGINAL-IRRITATION TEST OF EXPERIMENTAL SPERMICIDES

Active Ingredient[a], %	Code No.[b]	Total Irritation Counts per Rabbit[c] Rabbit Number 1	2	3	4	5	6	Average
0.5 Lauramidine hydrochloride	12	20	1	15	10	18	15	13
0.5 Methylene dithiocyanate	19	44	47	44	34	29	44	40
8.0 *t*-Dodecylthionona(ethyleneoxy)ethanol	20	4	4	2	4	0	0	2
		0	1	0	0	3	0	1
8.0 *t*-Tridecoxypoly(ethyleneoxy)ethanol	21	0	0	0	0	0	0	0
4.0 *t*-Nonylphenoxypoly(ethyleneoxy)ethanol	29	0	0	0	5	1	0	1
		2	5	5	0	1	2	3
		7	3	0	3	0	7	3
4.0 Octylresorcinoxynona(ethyleneoxy)ethanol	32	5	2	2	0	5	1	3
		0	0	0	1	1	2	1
2.0 N,N,N′-Tris(hydroxyethylpolyoxyethylene)-N′-octadecyl-1,3-propylenediamine acetate	37	7	8	6	0	6	6	6
0.25 Bis(*n*-butylphenyl)iodonium chloride	51	23	14	1	7	6	6	10
Blank	...	0	0	1	4	0	0	1

[a] The active ingredient was formulated in a jelly base containing 2.0% boric acid, 10.0% glycerol, 0.02% butyl *p*-hydroxybenzoate, and 3.0% gum tragacanth. The pH was approximately 4.5.

[b] The code numbers that were used in a previous publication (2) are given here for comparison purposes.

[c] The irritation count is a measure of the various degrees of erythema, exudate, and edema observed on the days following each of three daily applications of jelly. Each type of irritation was graded as a 1, 2, or 3 degree level, depending upon its severity. Erythema was considered the mildest type of irritation. Each degree of erythema (designated as *E*) was given an irritation count of 1; i. e., $E_1 = 1$, $E_2 = 2$, $E_3 = 3$. E_1 was characterized by a slight redness of the mucosa; E_2 was characterized by redness accompanied by occasional distinct blood vessels; and E_3 was characterized by a deep redness accompanied by numerous engorged blood vessels. The presence of an exudate, composed of pus and/or sloughed tissue, was considered more severe than erythema but less severe than edema. Each degree of exudate (designated as *X*) was given an irritation count of 2; i.e., $X_1 = 2$, $X_2 = 4$, $X_3 = 6$. X_1 was characterized by a few small globules of pus; X_2 was characterized by large globules of pus; and X_3 was characterized by large amounts of pus and/or sloughed tissue exuding from the vulva. Edema was considered the most severe type of irritation. Each degree of edema (designated as *ED*) was given an irritation count of 3; i. e., $ED_1 = 3$, $ED_2 = 6$, $ED_3 = 9$. ED_1 was characterized by a slight swelling of the vulva; ED_2 was characterized by a swelling of the vulva to nearly twice its normal size; and ED_3 was characterized by a swelling of the vulva to several times its normal size. The various irritation counts for the three days were totaled for each rabbit. (For example, a rabbit showing E_1 on the first day, E_2, X_2, and ED_1 on the second day, and E_3, X_3, and ED_3 on the third day would be given irritation counts of 1, 9, and 15 for each of the three days, respectively, making a total of 25.) The highest total irritation count per rabbit possible is 54.

TABLE II.—THERAPEUTIC INDEXES OF EXPERIMENTAL SPERMICIDES

Code No.	Maximum Nonirritating Concentration,[a] %	Minimum Spermicidal Concentration,[b] %	Therapeutic Index[c]
12	0.25	0.5	0.5
19	0.1	0.1	1
20	8.0	0.5	16
21	8.0	0.5	16
29	4.0	0.5	8
32	4.0	0.5	8
37	1.0	0.5	2
51	0.1	0.1	1

[a] The maximum nonirritating concentration is an approximation of the maximum concentration that will result in an average irritation count of less than 5 in the rabbit vaginal-irritation test.

[b] The minimum spermicidal concentration is the minimum concentration that will result in an instantaneous immobilization of sperm in the modified Brown and Gamble test (2).

[c] The therapeutic index is considered here as the ratio of the maximum nonirritating concentration to the minimum spermicidal concentration.

* Received November 14, 1958, from the Department of Obstetrics and Gynecology, Ohio State University College of Medicine, and Battelle Memorial Institute, Columbus, Ohio.

This study was sponsored by the Research Department, Holland-Rantos Company, Inc., New York 13, N. Y.

effective spermicides (2) using the rabbit vaginal-irritation test (1). Table II shows the therapeutic indexes of those materials.

Compound 51, for example, is five times as effective a spermicide as Compound 21; however, it has a much lower therapeutic index and thus would be much less satisfactory than Compound 21 as a contraceptive principle. The importance of using the rabbit vaginal-irritation test in conjunction with a spermicide test for evaluating spermicides is, therefore, made apparent.

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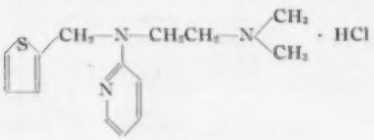
A Note on the Crystallography of Thenylpyramine Hydrochloride*

By HARRY A. ROSE and JOHN G. WILLIAMS

THENYLPYRAMINE HYDROCHLORIDE is a drug which is useful in the relief of allergic conditions. Data are presented here which permit the identification of this compound by crystallographic methods.

The chemical name of the compound is 2-[(2-dimethylaminoethyl)-2-thenylamino] pyridine and is represented by the following structural formula:



EXPERIMENTAL

Crystals suitable for crystallographic work may be obtained by recrystallization from isopropyl alcohol. The crystals are monoclinic plates lying on the 100 face and closed by the 011 faces. Occasionally small 210 faces may be seen.

TABLE I.—X-RAY DIFFRACTION DATA

Unit Cell Dimensions.	$a_0 = 27.35$ *A*, $b_0 = 10.38$, $c_0 = 10.96$ *A*
Formula Weights per Cell.	8
Formula Weight.	297.85
Density.	1.273 Gm./cc. (flotation); 1.281 Gm./cc. (X-ray)
Beta Angle.	96°
Axial Ratio.	$a:b:c = 2.635:1:1.056$
Space Group.	$C_2^5h - P2_1/n$

TABLE II.—OPTICAL CRYSTALLOGRAPHIC DATA

Refractive Indexes. (5893Å., 25°).	$\alpha = 1.592$, $\beta = 1.666$, $\gamma = 1.700$, β' (in 100) = 1.642
Optic Axial Angle.	$(-)\ 2V = 65°40'$ (calcd. from α, β, and γ)
Orientation.	$\beta \wedge c = 32°$ in acute β, $\gamma = b$

* Received March 28, 1959, from the Analytical Department, Eli Lilly and Co., Indianapolis 6, Ind.

The X-ray powder diffraction data were obtained using copper radiation and nickel filter with a camera 114.6 mm. in diameter. A wavelength value of 1.5405 *A* was used in the calculations. The indexing of the powder pattern was done on the basis of single crystal rotation patterns around both the *b* and *c* axis.

On heating, thenylpyramine hydrochloride melts in the range 164.5–166.5°. The melt does not crystallize on cooling.

TABLE III.—X-RAY POWDER DIFFRACTION DATA

d	I/I_1	*hkl*	*d* (calcd.)
8.26	0.03	210	8.25
7.56	0.17	011	7.52
6.80	0.13	400	6.80
6.41	0.03	211	6.39
5.71	0.07	410	5.76
5.24	0.33	$\bar{2}02$	5.21
4.87	0.50	202, 220	4.89, 4.85
4.69	0.07	021	4.69
4.54	0.13	600	4.53
4.39	0.13	221	4.37
4.15	1.00	610	4.15
4.04	0.13	402	4.05
3.74	0.50b	412, 022, $\bar{6}02$	3.76, 3.76, 3.68
3.56	0.17	222	3.56
3.41	0.13b	620, 422, 230	3.41, 3.40, 3.35
3.17	0.20	422	3.19
2.99	0.20	023	2.98
2.91	0.20	$\bar{8}12$	2.92
2.72	0.13	10, 00	2.72
2.62	0.07		
2.55	0.07		
2.50	0.07		
2.43	0.03		
2.40	0.07		
2.34	0.07		
2.29	0.03		
2.23	0.03		
2.13	0.07		
2.07	0.03		
2.04	0.03		
2.01	0.03		

The United States Pharmacopœial Convention, Inc.

ANNUAL FINANCIAL STATEMENT

Based upon the Report of the Auditor for the year ended December 31, 1958

STATEMENT OF INCOME AND EXPENSE

Income:			
Sales of pharmacopeias:			
Collections		$39,098.75	
Less—Decrease in account receivable		3,960.25	$ 35,138.50
Sales of reference standards:			
Collections		$41,964.18	
Add—Increase in accounts receivable and adjustment of prior years' collections		1,346.12	43,310.30
Interest on investments, less amortization of bond premiums of $22.65			6,839.85
Interest on savings deposits			6,489.61
Use of text by others			20.00
Miscellaneous			88.62
			$ 91,886.88
Expense:			
Revision		$95,722.15	
Less:			
Increase in inventory	$ 905.43		
Opening of employees' pension trust account	300.00	1,205.43	$ 94,516.72
Administration			10,507.14
Publications		$ 1,023.39	
Add—Decrease in inventory		9,603.88	10,627.27
Headquarters		5,772.12	
Add—Depreciation expense:			
Building	2,430.77		
Furnishings and equipment	4,146.68	6,577.45	12,349.57
			$128,000.70
Excess of Expense over Income			$ 36,113.82

STATEMENT OF CASH RECEIPTS AND DISBURSEMENTS (GENERAL ACCOUNT)

Receipts:			
Sales of pharmacopeias		$39,098.75	
Sales of reference standards		41,964.18	
Interest on investments		6,862.50	
Interest on savings deposits		6,355.21	
Use of text by others		20.00	
Miscellaneous		88.62	
		$94,389.26	

Disbursements:	Revision	Administration	Publications	Headquarters	Furnishings and Equipment	Total
Salaries	$57,416.77	$ 5,245.00	...	$1,675.00	...	$ 64,336.77
Meetings	5,567.87	115.21	...	...	...	5,683.08
Supplies	1,488.16	115.38	...	...	...	1,603.54
Postage and telegrams	1,642.69	50.33	...	...	...	1,693.02
Utilities	....	....	...	2,243.45	...	2,243.45
Repairs and maintenance	....	....	...	1,154.22	...	1,154.22
General	29,606.66*	4,981.22	$1,023.39	...	...	35,611.27
Insurance and taxes	....	....	...	699.45	...	699.45
Furnishings and equipment	....	....	...	...	$1,032.63	1,032.63
	$95,722.15	$10,507.14	$1,023.39	$5,772.12	$1,032.63	$114,057.43

* Retirement and Social Security	$11,502.78
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Promotion	794.90
Miscellaneous	524.78
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Professor of Pharmacognosy
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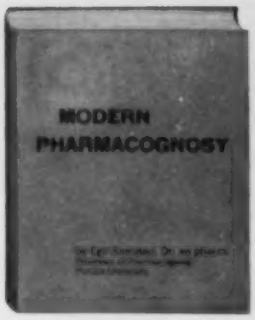

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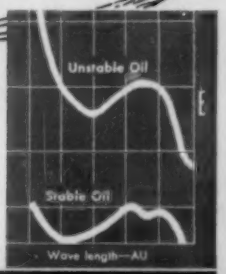

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